The Influence of Selol on the Expression of Oxidative Stress Genes in Normal and Malignant Prostate Cells

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Abstract. *Selol is a mixture of selenitriglycerides, obtained by the chemical modification of sunflower oil, which contain selenium at the +4 oxidation state. The aim of the present study was to describe the changes in the expression of genes related to oxidative stress caused by Selol in prostate cells: both normal (PNT1A) and malignant (LNCaP). The changes in gene expression in PNT1A and LNCaP cell lines under the influence of Selol were measured using a 96-well RT[2] Profiler ™ PCR Array: Human Oxidative Stress and Antioxidant Defense, which arrayed 84 genes functionally involved in the cellular oxidative stress response. Based on the obtained data, LNCaP cells exhibited a significantly lower potential for antioxidant defence when compared to PNT1A cells. The response of the malignant LNCaP cells to exposure to Selol was significantly different from that of the normal PNT1A cells, especially after 48 h of incubation. In the case of LNCaP cells, Selol causes down-regulation of the expression of many vital genes. Under in vitro conditions, the efficacy of Selol slightly changes with increasing concentration, but significantly increases when the incubation time is lengthened.*

The ability of malignant cells to acquire multidrug resistance, along with the powerful side-effects caused by chemotherapy are the driving forces behind the constant search for new, more efficient and selective therapeutic compounds.

One of these potential anticancer agents is Selol [Patent, Pol. PL 176530 (Cl.A61K31/095)], an organic selenium (IV) compound consisting of a mixture of selenotriglycerides obtained by the chemical modification of sunflower oil (1).

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Using the Ames test, Rahden-Staroń *et al*. demonstrated, that Selol has no mutagenic effect on strains of *Salmonella typhimurium* (2). Our own investigations into the cytotoxicity of 5% Selol showed that it is toxic to malignant HeLa cells (3) as well as HL-60 human promyelocytic leukaemia cells, both susceptible and multidrug resistant (4), dependent on dosage and incubation time. Work carried out with BJ normal human fibroblasts and PNT1A normal human prostate epithelial cells (data not published) revealed the significantly lower toxic effect of Selol on these cell lines when compared to malignant HeLa cells. It was also shown that due to being a mixture of triglycerides, Selol exhibits much lower levels of *in vitro* cytotoxicity when compared to sodium selenite, an inorganic selenium compound (3). This effect has also been confirmed through *in vivo* experiments (5).

Due to its antioxidant properties, as well as its presence as part of certain enzymes, selenium takes part in metabolic processes at the cellular level, protecting cellular membranes from free radicals, preventing lipid peroxidation, protein oxidation, and DNA and RNA damage, all of which may lead to a lowered risk of malignant changes taking place (6). On the other hand, a high intracellular selenium concentration leads to the formation of reactive oxygen species (ROS), which in turn leads to apoptosis due to the imbalance between ROS generation and the antioxidant potential of the cell (7).

As part of ongoing research into the mechanism of action of Selol, this article aims to describe the changes in the expression of genes related to oxidative stress caused by Selol in prostate cells, both normal (PNT1A) and malignant (LNCaP).

Prior to undertaking molecular analysis, the cytotoxic influence of 5% Selol on selected prostate cells was measured using the salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay).

The levels of gene expression were determined using real-time polymerase chain reaction (PCR) using a commercially available array containing 84 genes related to cellular

responses to oxidative stress. The array was selected based on the pro-oxidative properties of selenium described in the literature. It was assumed that the toxic properties of SeIol, observed in cytotoxic studies, are due in part to its ability to disrupt the cellular oxidoreductive balance, leading to the induction of apoptosis and subsequent cell death (7, 8).

A complex statistical analysis was carried out on genes which displayed changes in expression level, which then led to conclusions concerning the cellular processes taking place under the influence of SeIol.

Materials and Methods

Compounds and reagents. SeIol was synthesized at the Department of Analysis of Medicines at the Medical University of Warsaw, as described in the patent (1). A micellar solution of SeIol was used (based on lecithin, water and SeIol), with a declared selenium concentration of 5% (w/v).

The following reagents were used: phosphate buffered saline (PBS) without Mg^{2+} and Ca^{2+} ions (Institute of Immunology and Experimental Therapy, Wroclaw, Poland), sterile water (Polfa, Lublin, Poland), RPMI and fetal bovine serum (FBS) (Lonza, Verviers, Belgium), antibiotics: penicillin, streptomycin, amphotericin B (Lonza, Walkersville MD, USA), trypsin-EDTA and glucose (Sigma, St. Louis MD, USA), HEPES, sodium pyruvate and (Sigma, Irvine, USA), GeneMatrix Universal RNA/miRNA Purification Kit (EURx, Gdansk, Poland), RT^2 First-Strand Kit (Qiagen, Maryland, USA), RT^2 SYBR Green/ROX qPCR Master Mix (Qiagen) and RiboLock™ RNase Inhibitor (Fermentas, ABO, Gdansk, Poland).

Cell culture. The model system described in literature for investigating the properties of selenium is a pair of prostate cell lines, one normal (PNT1A) and one malignant (LNCaP) (9, 10). The LNCaP cell line was first derived from prostate metastases in the superclavicular lymph nodes (11). *In vitro* testing showed that this line has a doubling time longer than most malignant cell lines, of approximately 60 h. LNCaP cells present with morphology characteristic of malignant epithelial cells, including the expression of specific cytokeratins 8 and 18, as well as a lack of desmin and factor VIII (12). Due to high sensitivity and low adherence following splitting, they require a 48-hour incubation period to properly adhere to the culture flask. The PNT1A normal cell line was derived from the prostate of a 25-year-old man and immortalised by transfection with a plasmid containing the SV40 viral genome. They display a differentiated luminal prostate cell phenotype, expressing cytokeratins 8 and 18, as well as vimentin. The cell line makes a good model for analysing cellular processes, such as the proliferation of prostate epithelium. The doubling time for these cells in culture is approximately 25 h (13).

Both the PNT1A (ECACC, Salisbury, UK) and LNCaP (ATCC, Manassas, USA) cell lines were cultured at 37˚C with 5% CO_2, using RPMI with 10% FBS and 1% antibiotics mixture. In the case of the LNCaP cell line, 10 mM HEPES, 1 mM sodium pyruvate and 4500 mg/l of glucose were also used (as indicated by the ATCC). For all work concerning gene expression levels, 1×10^6 cells were introduced into 25-cm^2 cell culture flasks. Due to the difference in doubling times between the two cell lines, PNT1A cells were cultured for 24 hours and LNCaP cells were cultured for 48 h prior to exposure to the compounds of interest using the conditions described above, in order to reach a similar level of confluence (approx. 70%).

Incubation with SeIol. For the purposes of investigating gene expression in PNT1A and LNCaP cells, the concentration of SeIol was chosen based on a previously performed cytotoxicity assay (MTT). The concentration chosen was one that caused an approximately 30% reduction in the number of live LNCaP cells in culture following a 24-h incubation, *i.e.* 150 μM with respect to selenium. The same concentration was used for PNT1A cells. The impact of SeIol on gene expression was observed in both cell lines following 24 and 48 h of incubation. A culture of appropriate cells in medium was used as a control.

RNA isolation and quantification. Total RNA was isolated from cells using the GeneMatrix Universal RNA/miRNA Purification Kit. The isolation was performed in three independent replicates for each experimental system. The quantity and quality of isolated RNA was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). The isolated RNA was stored at −70˚C with added RiboLock™ RNase Inhibitor (Fermentas).

PCR array. The investigation of gene expression in the chosen experimental systems was carried out using RT^2 Profiler ™ PCR Array: Human Oxidative Stress and Antioxidant Defense (Qiagen). The efficiency of cDNA synthesis along with the quality of the resulting material were examined using RT^2 RNA QC PCR Array (Qiagen).

Measuring gene expression using real-time PCR. The experiment consisted of the following steps: a) Clean-up of the isolated RNA in order to remove contaminating DNA followed by cDNA synthesis using RT^2 First Strand Kit. b) Confirmation of the presence of cDNA in the samples using Human RT^2 RNA QC PCR Array. c) Quantitative analysis of gene expression in the samples using *RT^2 Profiler™ PCR Array: Human Oxidative Stress and Antioxidant Defense*, using an MX3005p cycler (Stratagene) and RT^2 SYBR Green/ROX qPCR Master Mix. d) Data analysis using the ΔΔCt method, using RT^2 Profiler PCR Array Data Analysis software version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Quantitative assessment of gene expression is usually carried out using real-time PCR, where gene expression is measured through quantitative and qualitative changes in mRNA caused by an external factor (14). The changes in gene expression in PNT1A and LNCaP cell lines under the influence of SeIol were measured using a 96-well RT^2 Profiler ™PCR Array: Human Oxidative Stress and Antioxidant Defense, which contained 84 genes functionally-involved with the cellular oxidative stress response. The array also contained 12 control genes, including five housekeeper genes for normalisation: Beta-2-microglobulin (*B2M*), Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), Ribosomal protein L13a (*RPL13A*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPHD*) and Actin beta (*ACTB*) as well as controls for genomic DNA contamination, reverse transcription repeatability (3 repeats) and PCR reaction repeatability (3 repeats).

In order to qualify for gene expression analysis, the genetic material had to fulfil conditions set by the provider of the array, *i.e.* a spectrophotometric assessment of the isolated RNA had to yield an A260:A230 ratio of at least 1.7 and an A260:A280 ratio of at least 2.0. The experimentally optimised RNA input for cDNA

synthesis was 1 μg. The efficiency of cDNA synthesis along with the quality of the resulting material was additionally controlled using an RT^2 RNA QC PCR Array. Three independent repeats were carried out for each experimental system, following which the results were analysed using RT^2 Profiler PCR Array Data Analysis software version 2.5 (Qiagen).

In the case of PNT1A, the genes used to normalize the results were: *B2M, HPRT1, RPL13A, GAPDH* and *ACTB*, in the case of LNCaP the genes used were: *B2M, HPRT1, GAPDH* and *ACTB*. The results gathered from comparing the PNT1A and LNCaP cell lines in their native state were normalised using the following genes: *HPRT1, RPL13A* and *GAPDH*. Both values for individual genes as well as their average, calculated for each experimental system, were within a margin of one cycle, which marks them as appropriate for normalising results gathered in each of the experiments.

The differences in response of the chosen cell lines to Selol, compared to controls consisting of the same cells without the added compound, were analysed using the $\Delta\Delta C_T$ method. Additionally, a comparative analysis was carried out on the results gathered from the PNT1A and LNCaP controls in order to establish the differences in the native expression of genes associated with the oxidative stress response. In the interpretation of these results, a minimum of a two-fold increase or decrease of gene expression compared to the control was treated as significant. The *p*-value for each fold change in expression was calculated using a Student's paired *t*-test. Results were assumed to be statistically significant for $p \leq 0.05$.

Results

Comparison of expression of genes associated with oxidative stress response in the native state in PNT1A and LNCaP cell lines. Upon comparing the gene expression profiles of PNT1A and LNCaP cells cultured in media without the addition of Selol, it was found that in LNCaP cells, the expression of 21 genes was lower and the expressoin of 14 genes was higher when compared to normal PNT1A cells (Table I). Amongst the genes with lower expression in LNCaP cells compared to normal cells were two genes which encode glutathione peroxidase-3 and -7: *GPX3* (9.09-fold reduction) and *GPX7* (470.41-fold), which play an important part in the anti-oxidation enzyme cascade, both containing selenium in their active sites in the form of selenocysteine (15). Reduced expression was also observed for other genes (fold change) encoding proteins with peroxidase functionality, *i.e. CYGB* (reduced by 5.61), *DUOX1* (by 4.27), *GPR156* (by 14.43), *PTGS2* (by 11.01), *PXDN* (by 14.57) and *TTN* (by 4.39). Amongst genes which encode enzymes with antioxidant properties, a lower expression in LNCaP cells was observed for the following genes: *TXNDC2* (12.59), encoding thioredoxin domain-containing protein-2 and *TXNRD1* (3.38), encoding thioredoxin reductase-1, which together form a thioredoxin – thioredoxin reductase complex, which plays an important part in protecting the cell from oxidative stress, as well as controlling the growth and proliferation of cells. Other reduced genes include *APOE* (64.05), encoding apolipoprotein E and *MT3* (2.76), encoding metallothionein 3. It was also observed that compared to normal PNT1A cells, LNCaP cells display a lower expression of the *SOD2* gene (2.90), encoding mitochondrial superoxide dismutase 2, whilst the expression of *SOD3*, encoding extracellular superoxide dismutase-3, was found to be 3.18-times higher. SOD is an important part of the cellular defence against the toxic activity of free oxygen radicals. It accelerates the dismutation of the superoxide radical $O_2^{\bullet}$. Mitochondrial SOD2 is considered to be the most important enzyme protecting cells from oxidative stress, as the structure most susceptible to free radical attack is the mitochondrion. The free radicals generated there damage the weakly protected DNA, which leads to mutations resulting in the formation of a energy deficiency in the cells and the possibility of malignant transformations occurring (16).

In LNCaP cells, four out of the five genes associated with peroxide metabolism (*CYBA, NCF2, NOS2* and *PREX1*) exhibited lower expression when compared to PNT1A cells. The greatest difference was observed for *CYBA*, responsible for encoding the alpha subunit of cytochrome *b-245*, which is virtually inactive in malignant cells reduced by <1000. This particular protein is responsible for generation of peroxides and phagocytosis.

An over 10-fold drop in expression was also observed for other genes associated with oxidative stress response, *i.e. AOX1* (15.83), encoding aldehyde oxidase 1 which takes part in the metabolism of reactive oxygen species (ROS: $O_2^{-\bullet}$, $HO_2^{\bullet}$, H_2O_2, $^{\bullet}OH$), as well as *PDLIM1* (7.86), *PRNP* (3.76) and *SCARA3* (146.13) genes.

Within the group of genes which were found to have a higher expression in LNCaP cells the one in PNT1A cells were two genes *PRDX3* (2.66) and *PRDX4* (2.76), responsible for encoding mitochondrial and cytosol peroxiredoxin 3 respectively, which reduce hydrogen peroxides and lipid hydroxides (17).

A higher expression was also recorded for three genes encoding enzymes which display peroxidase activity, *i.e. CAT* (6.54), *EPX* (3.63) and *TPO* (4.70). A much higher expression in LNCaP cells, when compared to PNT1A cells, was observed for the *ALB* gene (127.02), encoding albumin, which displays antioxidant properties, as well as for *BNIP3* (6.32), which encodes a pro-apoptotic mitochondrial protein, and *EPHX2* (4.22), which encodes cytoplasmic epoxide hydrolase-2 which takes part in ROS metabolism. A higher level of expression was also observed for three genes encoding kinases, *i.e. DGKK* (5.68) encoding diacylglycerol kinase, *NME5* (8.99) encoding nucleoside diphosphate kinase and *SGK2*, encoding serine-threonine protein kinase. Additionally, a higher level of expression in LNCaP cells was seen for the *SEPP1* gene (29.15), encoding selenoprotein P, which likely functions as protection against oxidants (18).

Table I. *Results from the Human Oxidative Stress and Antioxidant Defense PCR Microarray for LNCaP cells compared to PNT1A cells - without added Selol.*

Gene*	Description	*p*-Value	LNCaP/PNT1A**
Glutathione peroxidases			
GPX3	Glutathione peroxidase-3	0.007278	−9.09
GPX7	Glutathione peroxidase-7	0.003530	−470.41
Peroxiredoxins			
PRDX3	Peroxiredoxin-3	0.001096	+2.66
PRDX4	Peroxiredoxin-4	0.000862	+2.76
Other peroxidases			
CAT	Catalase	0,000173	+6.54
CYGB	Cytoglobin	0.000482	−5.62
DUOX1	Dual oxidase-1	0.020992	−4.27
EPX	Eosinophil peroxidase	0.000431	+3.63
GPR156	G-Protein-coupled receptor-156	0.001191	−14.43
PTGS2	Prostaglandin-endoperoxide synthase-2	0.019649	−11.01
PXDN	Peroxidasin homolog (Drosophila)	0.009363	−14.57
TPO	Thyroid peroxidase	0.007396	+4.70
TTN	Titin	0.014079	−4.39
Other antioxidants			
ALB	Albumin	0.006216	+127.02
APOE	Apolipoprotein E	0.000976	−64.05
MT3	Metallothionein-3	0.025712	−2.76
TXNDC2	Thioredoxin domain containing-2 (spermatozoa)	0.007697	−12.59
TXNRD1	Thioredoxin reductase-1	0.002185	−3.83
SOD			
SOD2	Superoxide dismutase-2, mitochondrial	0.000140	−2.90
SOD3	Superoxide dismutase-3, extracellular	0.000526	+3.18
Other genes involved in superoxide metabolism			
CYBA	Cytochrome b-245, alpha polypeptide	0.000192	−17053.47
NCF2	Neutrophil cytosolic factor-2	0.010442	−5.13
NOS2	Nitric oxide synthase-2, inducible	0.000036	−6.87
NOX5	NADPH oxidase, EF-hand calcium-binding domain-5	0.000971	+14.44
PREX1	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor-1	0.001106	−92.37
Other genes involved in reactive oxygen species (ROS) metabolism			
AOX1	Aldehyde oxidase-1	0.003376	−15.83
BNIP3	B-cell CLL/lymphoma 2/adenovirus E1B 1 19 kDa-interacting protein-3	0.007850	+6.32
EPHX2	Epoxide hydrolase-2, cytoplasmic	0.003837	+4.22
Oxidative stress response genes			
DGKK	Diacylglycerol kinase, kappa	0.003332	+5.68
NME5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	0.003147	+8.99
PDLIM1	PDZ and LIM domain 1	0.011408	−7.86
PRNP	Prion protein	0.003178	−3.76
SCARA3	Scavenger receptor class A, member-3	0.000513	−146.13
SEPP1	Selenoprotein P, plasma, 1	0.000004	+29.15
SGK2	Serum/glucocorticoid-regulated kinase-2	0.030576	+73.95

*Grouped according to function based on Qiagen listing. **−, Decrease; +, increase in gene expression.

Table II. *Results from the Human Oxidative Stress and Antioxidant Defense PCR Microarray for PNT1A and LNCaP cells, after treatment with Selol at 150 μM for 48 h.*

Gene*	Description	PNT1A/PNT1A control		LNCaP/LNCaP control	
		p-Value	Fold difference**	*p*-Value	Fold difference**
Glutathione peroxidases					
GPX2	Glutathione peroxidase-2	0,003766	+2.55	0,007688	+5.82
GPX5	Glutathione peroxidase-5	0,047941	+2.32	No change	No change
Other peroxidases					
DUOX1	Dual oxidase-1	0,004262	+2.35	No change	No change
EPX	Eosinophil peroxidase	0,049136	+4.30	No change	No change
GPR156	G-Protein-coupled receptor-156	0,020516	+3.04	No change	No change
PTGS1	Prostaglandin-endoperoxide synthase-1	0,013725	+2.29	0,035260	−2.45
PTGS2	Prostaglandin-endoperoxide synthase-2	0,045101	+2.28	0,031674	−2.27
TTN	Titin	0,006336	+3.29	No change	No change
Other antioxidants					
ALB	Albumin	0,012829	+3.71	0,045808	−2.29
MT3	Metallothionein-3	0,005833	+3.63	No change	No change
TXNRD1	Thioredoxin reductase-1	No change	No change	0,001456	+3.00
SOD					
SOD3	Superoxide dismutase-3, extracellular	0,004558	+2.25	No change	No change
Other genes involved in superoxide metabolism					
CCS	Copper chaperone for superoxide dismutase	0,006728	+2.25	No change	No change
NOS2	Nitric oxide synthase-2, inducible	No change	No change	0,014331	−2.70
NOX5	NADPH oxidase, EF-hand calcium-binding domain-5	0,020021	+2.37	No change	No change
Other genes involved in ROS metabolism					
EPHX2	Epoxide hydrolase-2, cytoplasmic	0,012714	+2.39	0,040221	−3.85
Oxidative stress response genes					
ANGPTL7	Angiopoietin-like-7	0.008396	+2.23	0.033668	−2.55
DGKK	Diacylglycerol kinase. kappa	0.027614	+3.64	0.028768	−3.59
OXSR1	Oxidative-stress responsive-1	-	-	0.015705	−2.37
SEPP1	Selenoprotein P plasma 1	0.000948	+2.70	No change	No change
SGK2	Serum/glucocorticoid-regulated kinase-2	0.037292	+12.51	No change	No change

*Grouped according to function based on Qiagen listing. **Fold difference from control PNT1A and LNCaP. −. decrease; +. increase in gene expression.

Changes in gene expression of PNT1A cells under the influence of Selol. Obtained data shows that following 24 h incubation with Selol at a Se concentration of 150 μM, PNT1A cells do not display changes in gene expression when compared to controls, apart from one gene: *SGK2*, which exibited a 3.6-fold rise in expression. Following a 48 h incubation, a rise in expression was observed for 10 genes encoding proteins which act as enzymatic antioxidant defence for the cell, along with an expression increase for eight genes encoding proteins which take part in ROS metabolism. Table II summarises the fold changes in gene expression for PNT1A cells in response to 48 h incubation with Selol, compared to a control consisting of the same cells cultured without Selol.

Amongst all the genes which displayed a change in expression following 48-h exposure to Selol, none of them showed a drop in expression when compared to the control. An over two-fold rise in expression was seen for two genes from the glutathione peroxidase family: *GPX2* (2.55) and *GPX5* (2.32), as well as six other genes encoding enzymes with peroxidase activity, namely *EPX* (4.30) encoding eosinophil peroxidase, *GPR156* (3.04) encoding G-protein coupled receptor 156, *TTN* (3.29) encoding titin, the largest known protein, *DUOX1* (2.35) encoding dual oxidase 1, as

well as *PTGS1* (2.29) and *PTGS2* (2.28), encoding prostaglandin synthase-1 and -2, respectively. In the group of genes encoding proteins other than peroxidases which take part in antioxidant defence, a higher expression was recorded for the *ALB* (3.71) and *MT3* (3.63) genes.

In the functional group of genes associated with ROS metabolism, following a 48-h incubation of PNT1A cells with Selol, a 2.25-fold increase in the expression of *SOD3*, along with two other genes involved in superoxide metabolism, namely CCS (2.25) encoding copper chaperone for *SOD1* and *NOX5* (2.37) was observed. Additionally, amongst genes associated with ROS metabolism, a 2.39 increase in expression was observed for the *EPHX2* gene.

In the group of genes encoding proteins whose activity is also associated with cellular response to oxidative stress, a 12.51-fold increase in the expression of the *SGK2* gene was observed, which is an almost 4-fold greater increase in expression compared to the control than that seen following 24 h of incubating PNT1A cells with Selol. A higher expression brought on by exposure to Selol was also observed for the *ANGPTL7* gene (2.23), which takes part in angiogenesis regulation, as well as for *DGKK* (3.64) and *SEPP1* (2.70).

Changes in gene expression of LNCaP cells under the influence of Selol. The data gathered show that following 24 h incubation with Selol at a Se concentration of 150 μM, LNCaP cells show increased expression for only two genes from the antioxidant group, GPX2 (4.32) and *TXNRD1* (2.67), along with a drop in expression of one gene from the family encoding proteins associated with peroxide metabolism, *NOS2*, encoding nitric oxide synthase-2 (3.02).

Data gathered following 48 h incubation of LNCaP cells with Selol (Table II) confirmed the rise in expression of *GPX2* (5.82) and *TXNRD1* (3.00), along with the drop in expression of NOS2 (2.70), when compared to controls. Increasing the incubation time to 48 h resulted in an increase in the fold change in expression when compared to the shorter incubation time.

Additional reductions in expression were seen in genes from the antioxidant family, namely *PTGS1* (2.45), *PTGS2* (2.27) and *ALB* (2.29). In the group of genes encoding proteins which take part in ROS metabolism, there was a 3.85-fold drop in the expression of the *EPHX2* gene. Decreased expression was also seen in *ANGPTL7* (2.55), *DGKK* (3.59) and *OXSR1* (2.37), encoding serine/threonine protein kinase OSR1, which reduces kinase activity in response to oxidative stress.

It is important to note that whilst the normal PNT1A cells displayed an increased expression of *PTGS1, PTGS2, ALB, EPHX2, ANGPTL7* and *DGKK* genes following 48 h incubation with Selol, the same incubation with malignant LNCaP cells resulted in decreased expression of all these genes.

Discussion

The main aim of the curret investigation was to understand the mechanism of action of Selol, an organic selenium (IV) compound containing selenotriglycerides. Due to the oxidative properties of selenium, the present work aimed to determine the impact of Selol on the expression profiles of genes associated with the oxidative stress response in human prostate cell lines: LNCaP (malignant) and PNT1A (normal).

Data found in literature show that malignant cells display significantly lower activity of certain antioxidant enzymes when compared to normal cells (19, 20). The work described here confirms these findings. LNCaP cells exhibited a significantly lower potential for antioxidant defence when compared to PNT1A cells, displayed by lowered expression of many proteins with peroxidase activity, lowered expression of the gene encoding mitochondrial superoxide dismutase (*SOD2*) as well as two genes encoding the enzymes of the thioredoxin-thioredoxin reductase complex (*TXNDC2* and *TXNRD1*). These genes are all included in the basic mechanism of cellular defence against ROS.

Following 24-h incubation with Selol, PNT1A cells displayed an increased expression (when compared to the control in PNT1A cells not incubated with Selol) of only one gene: *SGK2*. The expression of this gene increases rapidly when the cellular oxidoreductive balance is disturbed. The kinase encoded by this gene is activated through phosphorylation, which occurs as a response to the presence of H_2O_2 molecules in the cellular environment (21). The kinase takes part in regulating the membrane transport of sodium ions (*via* the epithelial Na^+ channel) (22). Following 48 h of incubation with Selol, a significantly larger number of genes displayed increased expression in PNT1A cells when compared to LNCaP cells. Eighteen genes from the antioxidant and ROS metabolism groups were activated, with no genes exhibiting down-regulation.

This reaction of normal cells to stimulation by high intracellular selenium concentrations (caused by Selol), clearly indicates the activation of a specific mechanism of defence against oxidative stress.

The response of the malignant LNCaP cells to exposure to Selol was significantly different from that of the normal PNT1A cells, especially after 48 h of incubation. Following 24 h of incubation with the Selol, LNCaP cells displayed increased expression of genes encoding glutathione peroxidase-2 and thioredoxin reductase-1, with no additional genes being up-regulated, even after 48 h of incubation. Eight genes, however, displayed down-regulation when compared to the control. These genes included *NOS2*, which encodes inducible nitric oxide synthase – an important enzyme responsible for synthesising nitric oxide from the nitrogen side chain of L-arginine in the presence of NADPH and molecular oxygen. The NO molecule plays an important

role in many biological processes, acting as a neurotransmitter and a vasodilator, as well as taking part in blood clotting, iron metabolism and the activation of cytotoxic macrophage activity (23, 24). It also takes active part in redox complexes, and can act either inhibit or induce apoptosis, by being present at low or high concentrations, respectively (25). The initial level of expression of this particular gene in malignant cells was almost 7-fold lower when compared to the of normal cells, which was down-regulated further when exposed to Selol. Down-regulation of *NOS2* expression must lead to a drop in the intracellular concentration of NO, which likely leads to cellular processes involving this molecule to come to a stop. It is worth noting that Selol had no impact on the level of expression of this gene in normal cells. Incubation of LNCaP cells with Selol also led to the down-regulation of genes encoding prostaglandin-endoperoxide synthase 1 and 2, also known as cyclooxygenase-1 and -2 (COX). Cyclooxygenase is a basic enzyme in the process of prostanoid synthesis, including prostaglandins, prostacyclins and thromboxanes (26), which have a significant impact on physiological and pathophysiological processes, infuding inflammation. The expression of the *EPHX2* gene was down-regulated. This gene encodes cytoplasmic epoxide hydrolase-2, which displays two types of enzymatic activity: epoxide hydrolase activity and phosphatase activity. The latter takes part in the metabolism of epoxides formed by arachidonic acid and other unsaturated fatty acids (27). Another interesting gene, in the context of the activity of Selol, which displayed down-regulated expression, was *ANGPTL7*, which encodes angiopoietin. Angiopoietins, along with growth factors, proteolytic enzymes and adhesion molecules, take part in processes such as angiogenesis, as well as the process of cellular response to oxidative stress (28). Down-regulation of expression of this gene in malignant cells is very favourable in the context of halting angiogenesis which accompanies tumour progression.

Whilst analysing the results of the gene array used in this investigation, it is worth mentioning that amongst those genes seen as part of the most important system of enzymatic cellular defence against oxidative stress (*SOD, GPx, TPx, CAT, TXNDC2, TRNRD1* and *TXNRD2*), PNT1A cells incubated with Selol displayed activation of only the *GPX3*, *GPX5* and *SOD3* genes (following 48 h of incubation), whilst LNCaP cells only displayed activation of *GPX2* and *TXNRD1* (following both 24 and 48 h of incubation).

These changes in gene expression brought on by incubation with Selol may be caused by the fact that the biochemical pathways activated by the cells in response to external stimulation are extremely dynamic, with the changes not being constant throughout time. Such data are often termed *snapshot* gene activity data, based on the fact that the measurement of gene expression levels occurs at only one timepoint (29). The gene expression profiles described above are indicative of the state of the cells at 24 and 48 h of incubation with Selol. The delayed cellular response is most likely caused by the fact the Selol, due to its complex structure and micellar formulation used in *in vitro* work, requires a long time to pass through the cellular membrane and to be subsequently metabolised, resulting in the formation of active metabolites, including ROS (2). The expression of the SGK2 gene in PNT1A cells was almost 4-fold higher following 48 h of incubation when compared to 24 h.

This would suggest that if both cell lines activate enzymes that take part in first-line defence against oxidative stress caused by Selol, then it most likely takes place in less than 48 h. After such a length of time, the cell is required to activate other defence mechanisms, most likely due to exhausting certain components of the first-line system, such as molecules taking part in reduction reactions. Based on the data gathered, the next line of cellular antioxidant defence appears to be much more efficient in PNT1A cells than it is in LNCaP cells. Compared to the response displayed by the malignant cell line, the normal cell line activated not only glutathione peroxidase 2 and 5, but also other genes encoding proteins with peroxidase functions, along with the *ALB* and *MT3* genes. Albumin and metallothionein proteins are part of the non-enzymatic antioxidant system. Along with antioxidant enzymes they form a successful line of defence against oxidative stress (30).

Based on obtained data, it is possible to ascertain that the greater cytotoxic impact of Selol on LNCaP cells is caused primarily by the lower efficiency of the antioxidant defence system of these cells when compared to PNT1A cells, which was clearly shown by comparing the gene expression profiles of both cell lines in their native state.

Having a significantly lower antioxidant defence potential, LNCaP cells may be more susceptible to the induction of apoptosis due to the presence of ROS compared to PNT1A cells.

Under *in vitro* conditions the effecacy of Selol slightly changes with increasing concentration, but significantly increases when the incubation time is lengthened, allowing it to pass through the cellular membrane and be subsequently converted to active metabolites.

Taking into account the gene expression data gathered, it appears that in the case of LNCaP cells, Selol causes down-regulation of expression of many vital genes, which suggests there may be other mechanisms of action for this compound, outside of its pro-oxidative one. This activity may be due to the structure of the Selol molecule, which contains unsaturated fatty acid bonds, as well as the fact that selenium is required by many seleno-dependent enzymes.

Conflicts of Interest

None declared.

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