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The influence of an organic selenium (IV) compound on progression of tumour induced using prostate cancer cells and gene expression connected to the oxidative stress response

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ABSTRACT

Analyse the influence of Selol on progression of tumour and the changes in gene expression, connected to the cellular oxidative stress response, in cells taken from tumours induced in immunodeficient mice through subcutaneous inoculation with malignant LNCaP prostate cells. Based on the PSA levels (at 5 weeks following inoculation of LNCaP), the mice were divided into three groups. Selol and a placebo were given for the following 3 weeks. After this time, the animals were sacrificed, blood was taken (PSA) and tumours were isolated. RNA from samples was analysed using Real-Time PCR. Treating mice with Selol (21 days at a dose of 17 mg/kg of b.m.) resulted in a drop in the rate of body mass reduction and stopped the increase of plasma PSA levels. The fold changes in expression of the genes, in cells derived from tumours, treated with Selol, in the array, apart from 1 (*NME5*), failed to exceed the required 2-fold change. Treating tumour-bearing mice with Selol resulted in a reduced rate of body mass loss and halted the increase in plasma PSA concentration, then compared to mice receiving the placebo. The expression of genes, involved in oxidative stress following treatment with Selol, in LNCaP cells, didn't change.

Keywords: Selol, LNCaP, Real-Time PCR

INTRODUCTION

Malignant tumours are a problem with a high degree of social impact. The International Agency for Research on Cancer estimates that in 2008 approximately 12.7 million people were diagnosed with cancer and 7.5 million people died due to cancer. In Europe 1.7 million people are diagnosed every year, with approximately 962 000 deaths. In Poland approximately 150 000 people are diagnosed and 93 000 die of cancer every year [1-3]. Despite the vast amount of effort being put into researching the causes of malignant disease and finding effective treatments, "the issue of cancer" remains unsolved. This is mainly because of the fact that cancer isn't a single disease, with tumour cells often developing therapeutic resistance during treatment. The aim of a large number of research teams is finding new therapeutic agents which will be more effectively and more selectively. At present, a number of research centres are investigating an organic selenium (IV) compound called Selol (Patent, Pol. PL 176530 (Cl.A61K31/095)), a mixture of selenotriglycerides obtained by the chemical modification of sunflower oil (Fig.1) [4]. Despite various investigations being carried out towards understanding the function of this element in the human body, it's exact mechanism of action remains unknown. Work carried out using normal and malignant human cell lines showed that Selol displayed significantly lower toxicity towards BJ normal human fibroblasts and PNT1A normal human prostate cells than towards malignant HeLa human cervical cells [5] and LNCaP human prostate cells [6]. It has also been shown that as an organic selenium

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compound, Selol causes significantly less toxicity than sodium selenite - a compound which has been certified for use in both nutrition and treatment [5,7]. Gene expression studies showed that Selol caused a more significant increase in the expression of genes responsible for the oxidative stress response in normal PNT1A cells than in malignant LNCaP cells [7]. This confirmed published findings which showed that malignant cells have significantly lowered activity of certain antyoxidation enzymes when compared to normal cells [8,9].

Literature data shows that selenium displays various forms of activity in cells, depending on its chemical form and dose [10]. In the context of antitumour prophylaxis, antioxidative properties are most commonly described. As a component of glutathione peroxidase, selenium takes part in the process of removing reactive oxygen species (ROS) from the cell, which protects it from potential DNA damage. It has been shown that a low cellular level of selenium is connected to a lowered glutathione peroxidase activity [11]. Additionally, selenium takes part in many biological processes through selenium-dependent enzymes, which indicates a possibility of using it as a preventative measure against various ailments connected to selenium insufficiency, or those tied to an increased level of reactive oxygen species (ROS) in cells [12].

The second avenue of selenium activity concerns its pro-oxidative properties and stems from the activity of metabolites created from selenium compounds during the biosynthesis of selenoproteins [13]. It is suggested that selenium is capable of inactivating biochemically important enzymes containing thiol, through oxidation and production of ROS, leading to apoptosis in mutated cells, but not in normal cells [11]. This sort of selenium activity, however, is seen at significantly higher concentrations than those achieved as a result of regular nutrition. Taking into account the significantly lower toxicity of Selol, compared to sodium (IV) selenite, it appears that Selol could potentially be used as an adjuvant for tumour therapy [5,7,11,14]. The aim of the work described here was analyse the influence of Selol on progression of tumour and the changes in gene expression, connected to the cellular oxidative stress response, in cells taken from tumours induced in immunodeficient mice through subcutaneous inoculation with malignant LNCaP prostate cancer cells. The levels of gene expression were measured using Real-Time PCR using a commercially available array containing 84 genes functionally connected to the cellular response to oxidative stress.

MATERIALS AND METHODS

Tested compound: Selol was synthesized at the Department of Analysis of Medicines at the Medical University of Warsaw as described in patent number Pol. Pl 176530 (Cl. A61K31/095) [4]. A micellar solution of Selol was used (consisting of lecithin, water and Selol) with a declared selenium concentration of 5% (w/v).

Reagents PBS (Phosphate Buffered Saline) without Mg^{2+} and Ca^{2+} ions (Institute of Immunology and Experimental Therapy), trypsin/EDTA (Lonza), RPMI (Lonza), Fetal Bovine Serum (FBS) (Lonza), antibiotics: penicillin/streptomycin/amphotericin B (Lonza), HEPES (Sigma), sodium pyruvate (Sigma), glucose (Sigma), GeneMatrix Universal RNA/miRNA Purification Kit (EURx), RT^2 First Strand Kit (Qiagen), RT^2 SYBR Green/ROX qPCR Master Mix (Qiagen), RiboLock™ RNase Inhibitor (Fermentas), Matrigel (BD Biosciences), Roche Elecsys Total Prostate-Specific Antigen (PSA) Assay using 1010 and 2010 immunoassay analyzers (Roche Diagnostics Corporation).

Cell culture: Human LNCaP malignant prostate cells (ATCC) were cultured in 5% CO_2 at 37°C in RPMI with added 10% FBS, 1% antibiotics mixture, 10mM HEPES, 1mM sodium pyruvate and 4500 mg/L glucose.

Laboratory animals: Immunodeficient NSG mice (NOD.Cg-Prkdc/scidIL2rg), sexually mature males aged approx. 12 weeks were kept on a standard diet (animals were kept at the Centre for Postgraduate Medical Training in Warsaw, following approval from the IV Local Ethics Committee for Animal Experimentation in Warsaw, resolution nr 33/2009 on 01.04.2009).

Tumour induction: Based on pilot studies a procedure for preparing and inoculating LNCaP cells into mice was developed. The optimal number of LNCaP cells for a single inoculation was 5×10^6 / mouse. Cells were suspended in Matrigel and injected subcutaneously into the shoulder, using a needle with a 25 G diameter. Time required for tumour growth: 4 - 5 weeks.

Experimental protocol: Using the procedure described above, 30 mice were inoculated with LNCaP cells. After 5 weeks, 150 µL of blood was taken from the tail of each mouse in order to estimate the size of the tumour. Following collection, the blood was centrifuged, after which the plasma was removed and passed on to a lab at the Medical University of Warsaw for PSA level analysis by immunodetection. Using the results

obtained from the PSA assay the mice were divided into three groups: with high (1), medium (2) and low (3) PSA levels. In each group 5 mice were selected for dosing (subgroups S1, S2, S3), and 5 were used as controls (subgroups K1, K2, K3).

Each animal received for a period of 3 weeks a single daily oral dose of either Selol (at a dose of 17 mg/kg b.m., based on results of investigating *in vivo* toxicity of Selol (data not published), or placebo in the form of sunflower oil (in the case of the control group).

Throughout the duration of the experiment, the animals were weighed and observed for changes in appearance and behaviour twice a week.

At the end of the 3 week period the animals were sacrificed, after which their blood was collected for analysis of PSA levels and their tumours were isolated for gene expression analysis. The tumours were weighed, after which they were divided into small fragments and snap frozen in liquid nitrogen, after which they were stored at -70°C.

RNA isolation and quantification: Based on pilot studies, the kit chosen to isolate genetic material (RNA) from the tumour tissue was the GeneMatrix Universal RNA/miRNA Purification Kit. The amount of tissue used for a single RNA isolation was 20 mg. The tumour fragment was homogenised in liquid nitrogen through the use of a mortar and pestle, after which it was lysed using a buffer supplied in the kit. The remainder of the isolation was carried out following the manufacturer's instructions.

Genetic material was isolated from 4 tumours taken from control group mice (untreated) and 4 tumours taken from dosing group mice (which received Selol). The quality and quantity of isolated RNA was measured using a NanoDrop spectrophotometer (Thermo Scientific). The average RNA isolation efficiency was 765 µg/mL. In order to qualify for further testing, samples needed to have an A_{260}:A_{230} ratio of at least 1.7 and an A_{260}:A_{280} ratio of at least 2.0. RNA samples that met these requirements were stored at -70°C with added RiboLock™ RNase Inhibitor.

PCR Array: Gene expression analysis was carried out on cells isolated from tumour tissue using a commercial gene array containing genes involved in the oxidative stress response: RT^2 Profiler TM PCR Array: Human Oxidative Stress and Antioxidant Defence (Qiagen). The efficiency and quality of cDNA synthesis was assessed using the RT^2 RNA QC PCR Array (Qiagen). The genes used in the array are listed in Table 1.

Measuring gene expression using Real-Time PCR: The experimentally optimised initial amount of RNA used for each round of cDNA synthesis was 1µg. The protocol consisted of the following steps: a) purification of isolated RNA to remove remaining DNA, followed by cDNA synthesis using the RT^2 First Strand Kit, b) confirmation of the presence and quality of cDNA in resulting samples using the Human RT^2 RNA QC PCR Array, c) analysis of gene expression of the samples using the RT^2 Profiler™ PCR Array using the MX3005p system (Stratagene) and the RT^2 SYBR Green/ROX qPCR Master Mix, d) analysis of results with the $\Delta\Delta C_t$ method using the RT^2 Profiler PCR Array Data Analysis software version 3.5, available on the following website: (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Statistical analysis: Statistical analysis was carried out using the SYSTAT software version 13.00.05 for Windows (Systat Software Inc., IL, USA). Results were statistically verified using the Mann-Whitney U test and Pearson correlation analysis. The level of statistical significance was set at $p \leq 0.05$.

RESULTS

The first phase of the investigation involved determining the procedure of inducing tumours in immunodeficient NSG mice by inoculating them with LNCaP cells. Two pilot experiments and one complete test were carried out. All the animals were monitored throughout the duration of the experiment. Following inoculation with LNCaP cells, all mice displayed a reduction in body mass (Table 2). Following separation into groups based on PSA1 concentration, the average mass for each group was 29.2 g. After 3 weeks of treatment with either Selol or placebo, the average mass, after subtracting the mass of the tumour, was 28.4 in the "Selol" group and 27.1 in the control group. This means that during the second part of the experiment (between weeks 5 and 8), the average body mass of the animals dropped by 0.8 g, i.e. 2.7%, in the Selol-treated group, and by 2.1 g, i.e. 7.2%, in the control group. This calculation shows that the animals receiving Selol experienced a slower reduction in body mass compared to the animals receiving the placebo, a difference that was statistically significant ($p<0.05$). Furthermore, the mice in the "Selol" group displayed a marginally higher mobility than the control mice at the end of the experimental period. Using of the developed procedure allowed to obtain subcutaneous tumors. 5 weeks after inoculation with LNCaP cells 20 mice presented with darkening of the skin at the injection site. The presence of induced tumours

was confirmed by measuring the concentration of PSA, which was elevated for all 30 mice, compared to mice that had not been injected with malignant cells, whose PSA concentration was below 0.003 ng/mL. Due to the tumours not growing uniformly amongst the experimental population, the animals were divided into groups based on PSA concentration at week 5 (Table 3, PSA1 values): 1) PSA: 18 - 62 ng/mL; 2) PSA: 7 - 14 ng/mL; 3) PSA below 7 ng/mL. In one case (mouse nr 24) levels of PSA could not be measured - this was caused by an insufficient quantity of material being gathered for analysis. Based on overall performance the animal was qualified into group S1.

Following 3 weeks of treating the animals with either Selol or placebo the experiment was ended and blood and tumours were collected from all animals. Figure 2 shows a mouse with a visible mass around its right shoulder as well as the tumour following isolation. Although the tumours in different mice were variable in size, all displayed a high degree of vascularisation. In the case of large tumours, a yellow-tinged exudate was observed on the inner side of the skin directly adjacent to the tumour tissue. The appearance of the tumours was varied in both experimental groups. Tumours treated with Selol were characterised by darker colouring, as well as a partly gelatinous internal structure, as opposed to the placebo-treated tumours which were compact and solid.

Table 3 summarises the mass and corresponding PSA2 level for each individual tumour. A correlation was found between the size of the tumour and the PSA concentration (R^2= 0.9287; $p<0.001$) (Fig. 3). Comparing the increase in PSA concentration in both groups of animals showed that the average ratio of PSA2 to PSA1 was 4.7 and 7.0 for the Selol treated and placebo treated groups respectively. This difference was found to be statistically significant ($p<0.05$). Taking into account the strong correlation present between PSA concentration and tumour size, it can be concluded that the lower fold change in PSA levels in the Selol treated group indicated a slowed tumour development in this experimental group. The analysis of *in vivo* gene expression using tumours induced by LNCaP malignant prostate cells was carried out in order to investigate whether the presence of selenium, derived from Selol, has an effect on the expression of genes connected to the cellular response to oxidative stress.

The analysis was carried out using a 96-well RT^2 Profiler™ PCR Array: Human Oxidative Stress and Antioxidant Defense array, containing a set of 84 genes functionally involved in cellular oxidative stress mechanisms (Table 1). Apart from the functional genes, the array also contained 12 control genes, including 5 basic metabolism genes which were used for normalisation: B2M, GPRT1, RPL13A, GAPHD, ACTB, and controls for genomic DNA contamination, reverse transcription reproducibility (in triplicate) and PCR reproducibility (also in triplicate).

Gene expression profiles were generated for cells taken from 4 tumours from control group mice (nr 5, 13, 16 and 27) and 4 tumours from "Selol" group mice (nr 2, 7, 8 and 12). The animals were chosen based on their placement in the various groups described: 2 mice were taken from both subgroups K1 and S1 (large tumours), 1 mouse was taken from both K2 and S2 (medium tumours), and, due to the lack of sufficiently sized tumours in K3, 1 additional mouse from K2 was taken along with 1 mouse from S3 (small tumours) (Table 3).

Table 4 summarises the C_T values measured for the normalisation genes for both the treated arm and the control arm of the experiment. Single genes, as well as their average calculated for each experimental arm, were all within 1 cycle of each other, which made them appropriate for use in normalising the data gathered in the various experiments.

The purpose of the statistical analysis of the gathered data was to find genes whose changes in expression patterns would present at least a 2-fold difference compared to an appropriate control. The results were summarised in Table 5.

Based on the data gathered, there appears to be no significant changes in gene expression in cells derived from tumours taken from mice treated with Selol, when compared to those treated with placebo (sunflower oil) which were used as a control. The fold changes in expression of the genes in the array, apart from 1 (*NME5*, 2.34 fold drop in expression compared to control), failed to exceed the required 2-fold change. It is worth noting that for all the genes in both experimental groups, the variability of the C_T value (the PCR reaction cycle at which the fluorescence value for a given gene exceeded a certain threshold) had a maximum value of 6.51%. This means that the results gathered for various animals in the same group were highly reproducible. Due to the fact that the tumours were induced in mice using human malignant prostate cells (LNCaP), each expression analysis was accompanied by a PCR product melting curve analysis after the PCR ran its course (following 40 cycles of amplification). No non-specific reaction products, whose presence could have potentially been a result of interference

caused by the presence of murine RNA in the samples, were found for any of the oxidative stress genes in the array.

DISCUSSION

The work described here is a continuation of research carried out between 2009 -2012 and is a pilot study, on the in vivo level, in seeking the Selol mechanism of action. Selol is a complex organic compound containing selenium at the +4 oxidation state, without any equivalent being described in literature so far. Various foods contain selenium, but its oxidation state is +2, usually in the form of selenomethionine and selenocysteine. Only a small proportion of the selenium acquired from these sources is incorporated into enzymatic active sites, most often in a non-specific manner, which contributes to the lack of biological activity of proteins involving selenium [15]. A compound containing selenium at the +4 oxidation state which is often used in experimental models is sodium selenite, but its high toxicity (in rats LD_{50}: 3.5 mg/kg b.m.) prevents it from being safety used as a supplement [16]. As opposed to selenites, synthetic organic selenium derivatives, such as Selol, allow the administration of significantly higher doses, making it possible to achieve required biological activity, whilst avoiding toxicity [17]. The antioxidative properties of selenium are well documented [18], however some publications contain hypothesise about the pro-oxidative properties and resulting anti-tumour activities of selenium compounds [11,19]. This hypothesis is the basis of the work carried out over the last few years, investigating the antitumor activity of Selol.
Initial *in vivo* work led to the optimal conditions for inducing tumours in immunodeficient NSG mice (NOD Cg-Prkdc/scidIL2rg). The volume and method of inoculation was found to be similar to those found in the literature [20,21]. Following injection with LNCaP human prostate cancer cells, the mice were under observation for changes in body mass as well as mobility. During the first part of the experiment - waiting for the tumours to develop (5 weeks), a loss in body mass was observed for all the animals. During the second part, following the administration of the first dose of either Selol or placebo, a difference in the rate of body mass reduction was observed between the two groups. The mice receiving Selol displayed a lower rate of body mass loss than the mice receiving the placebo. This observation may indicate that the process of tumour development in mice receiving Selol was stalled, leading to a slow improvement of their overall state. The observed increase in mobility might also be indicative of this process taking place.

Levels of PSA were measured twice, after 5 and 8 weeks following inoculation with LNCaP cells, and it was found that the blood plasma levels were increased for all animals. A correlation was found between the mass of isolated tumours and plasma PSA levels, which confirmed the usefulness of PSA as a marker for monitoring the development of LNCaP induced tumours. The data gathered was consistent with the findings of Lim et al. regarding the development of androgen-dependent human prostate cancer cells in nude mice [22].

The data gathered on fold changes in PSA level showed that the group receiving Selol had a smaller increase (4.7 fold) compared to the control group receiving placebo (7.0 fold). As was the case with the stalled body mass loss, this may indicate the beneficial effects of Selol on preventing tumour progression. Similar observations were made by a group investigating the effect of methylselenocysteine on the development of tumours in mice inoculated with LNCaP cells [21], where the rate of development of treated tumours was significantly slower after 2 weeks compared to the control group receiving PBS. It was also found that the mechanism of action of methyselenocysteine was linked to a drop in expression of the androgen receptor (AR) gene and the connected PSA gene.

Further confirmation of the cytotoxicity of Selol towards tumour cells can be found in the appearance of the tumours isolated from Selol treated mice. The tumour tissue was visibly darker, with the internal structure being gelatinous in places, as opposed to the compact and solid tumour tissue isolated from mice receiving the placebo.

The gene expression data gathered are not spectacular, because showed no difference between cells isolated from tumours treated with Selol and control tumours treated with sunflower oil. This means that at the end of the protocol, when the animals were sacrificed, the tumour cells treated with Selol (dosed for 3 weeks, once a day, single dose of 17 mg/kg b.m.) showed no indication of oxidative stress defence.

Based on the results of *in vitro* analysis [6, 23] and taking into account the duration for which the animals were given Selol (21 days), it is possible that ROS caused further changes in the cells, which led to the deterioration of an already impaired defence system for oxidative stress. It is possible that the LNCaP cells reached a state where they could no longer "defend" themselves, which is why there was no observable change in the expression of genes found in the array.

Another explanation for the results found could be the experimental protocol, where mice were given Selol once a day and the last dose was given on the penultimate day of the protocol. It is likely that at the point of tumour isolation (approx. 24 hours after the last dose of Selol) the animal and tumour tissue had already equilibrated, which is why the cells did not display any indication of active defence. It is possible that soon after receiving the dose of Selol there would be an oxidoreductive imbalance leading to a temporary increase in gene expression, indicating a defensive response to the damaging ROS.

An independent analysis using atomic absorption spectroscopy, carried out by a team at the Medical University of Warsaw, confirmed that the tumour tissue taken from mice treated with Selol contained 3.48 times more selenium than tumour tissue taken from mice treated with the placebo. The tissues contained, on average, 2.82 ± 0.19 µg selenium/g of tissue and 0.81 ± 0.06 µg selenium/g of tissue in animals treated with Selol and placebo respectively. It was also found that the livers of both healthy and tumour-carrying animals treated with Selol contained 5 times more selenium than those from animals treated with placebo. This shows that the selenium derived from Selol, following oral application, reached both vital organs and tumour cells [data not yet published], therefore the lack of response from oxidative stress response genes was not caused by a lack of selenium in the cells.

The fact that selenium reached the cells in the tumour indicates that it was active, with the lack of changes in gene expression likely caused by one of the reasons outlines above.

It is important to note that the gene expression data gathered, though not useful in the context of discussing genes which exhibit changes in expression after exposure to Selol, provide new valuable information with regards to planning effective treatment.

The data gathered so far on the *in vitro* activity of Selol, indicating an increased expression of certain genes connected to the cellular oxidative stress response [6] and increase in the oxidoreductive potential of cell derived from tumours isolated from mice treated with Selol, combined with a significant decrease in the concentration of reduced glutathione [data not yet published], indicate a pro-oxidative mechanism of action of Selol within cells at the concentrations investigated, as well as an increased cytotoxic activity towards malignant cells.

In the course of the *in vitro* investigations, during cell culture, the Selol derived selenium was present in the growth media throughout the entire incubation time, which is why the magnitude of its effect was dependent on both the incubation time and concentration. The cytotoxic activity of Selol towards malignant cells, resulting in the induction of apoptosis, was clearly noticeable as soon as after 48 hours of incubation [5,6]. In the case of *in vivo* conditions, there is a living organism involved, with metabolic processes taking place, and a single dose of the compound undergoes various changes, which finally result in metabolites.

The data gathered suggest that in order to achieve the desired pharmacological effect, as a result of the pro-oxidative activity of Selol, it would be necessary to increase the frequency of administration and/or increase the concentration administered, which would results in a more frequent / more durable presence of a state of oxidoreductive imbalance, which would, as a result of imperfect defence mechanisms, likely lead to damage to cellular structures and subsequent induction of apoptosis.

Taking into account the structure of the Selol molecule (the presence of unsaturated fatty acids), as well as the fact that selenium is part of various selenodependent enzymes, it is possible that this compound may have other mechanisms of anti-tumour activity aside from its pro-oxidative properties. The conclusions outlined above require confirmation through further study.

CONCLUSIONS

A correlation was found between the concentration of PSA in mouse blood plasma and the size of the tumour resulting from inoculation with LNCaP cells. Following 21 days of treating tumour-bearing mice with Selol, at a dose of 17 mg/kg of body mass, 24 hours after the last dose there was not detectable change in the expression of genes connected to the cellular oxidative stress response. Treating tumour-bearing mice with Selol over a period of 21 days, at 17 mg/kg b.m., resulted in a reduced rate of body mass loss and halted the increase in plasma PSA concentration, then compared to mice receiving the placebo. Furthermore, mice from the "Selol" group displayed marginally higher mobility than the control mice at the end of the protocol. The expression of genes connected to the cellular oxidative stress response, following treatment with Selol, in cells taken from tumours induced in mice, was weak.

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Conflict of Interest Statement: None declared.

Table 1: Genes found in the *RT² Profiler ™ PCR Array: Human Oxidative Stress and Antioxidant Defense* array divided into functional groups.

No.	Functional group	Gene name
ANTIOXIDANTS:		
1	Glutathione peroxidases	GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, GPX7, GSTZ1
2	Peroxiredoxins	PRDX1, PRDX2, PRDX3, PRDX4, PRDX5, PRDX6
3	Other peroxidases	CAT, CSDE1, CYGB, DUOX1, DUOX2, EPX, GPR156, LPO, MGST3, MPO, IPCEF1, PTGS1, PTGS2, PXDN, PXDNL, TPO, TTN
4	Other antioxidants	ALB, APOE, GSR, MT3, SELS, SOD1, SOD3, SRXN1, TXNDC2, TXNRD1, TXNRD2
GENES INVOLVED IN OXYGEN FREE RADICAL METABOLISM		
5	Superoxide dismutases	SOD1, SOD2, SOD3
6	Other genes involved in superoxide metabolism	ALOX12, CCS, CYBA, DUOX1, DUOX2, GTF2I, MT3, NCF1, NCF2, NOS2, NOX5, PREX1, PRG3
7	Other genes involved in oxygen free radical metabolism	AOX1, BNIP3, EPHX2, MPV17, SFTPD
8	Genes responsible for oxidative stress	ANGPTL7, APOE, ATOX1, CAT, CCL5, CSDE1, CYGB, DGKK, DHCR24, DUOX1, DUOX2, DUSP1, EPX, FOXM1, GLRX2, GPR156, GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, GPX7, GSS, KRT1, LPO, MBL2, MPO, MSRA, MTL5, NME5, NUDT1, OXR1, OXSR1, PDLIM1, IPCEF1, PNKP, PRDX2, PRDX5, PRDX6, PRNP, RNF7, SCARA3, SELS, SEPP1, SGK2, SIRT2, SOD1, SOD2, SRXN1, STK25, TPO, TTN, TXNRD2

Table 2. Summary of the changes in body mass (M) of the animals [g] throughout the protocol. Day 1 - start - inoculation with LNCaP cells (M1). Week 5 - separation into groups based on recorded PSA values and start of Selol / sunflower oil (placebo) dosing (M2). Week 8 - end of protocol (M3* - mass following subtraction of the mass of the isolated tumour).

Group 1 / large tumours							
Control – K1				**Selol – S1**			
Mouse no.	**Week 1**	**Week 5**	**Week 8**	**Mouse no.**	**Week 1**	**Week 5**	**Week 8**
	M1	**M2**	**M3***		**M1**	**M2**	**M3***
5	30.3	28.8	27.6	2	27.2	22.8	21.9
27	30.2	26.4	25.6	8	28.7	26.9	23.9
3	29.3	25.9	21.5	18	33.6	32.9	32.4
26	32.7	29.7	27.2	19	33.1	32.1	27.9
6	30.3	29.4	28.5	24	33.2	31.3	30.3
Xavg	*30.6*	*28.0 (M2/M1: 91,5%)*	*26.1*	*Xavg*	*31.2*	*29.2 (M2/M1: 93,6%)*	*27.3*
Group 2 / medium tumours							
Control – K2				**Selol – S2**			
Mouse no.	**Week 1**	**Week 5**	**Week 8**	**Mouse no.**	**Week 1**	**Week 5**	**Week 8**
	M1	**M2**	**M3***		**M1**	**M2**	**M3***
16	29.4	28.6	25.3	1	25.8	23.4	22.2
9	30.9	29.4	27.4	7	29.2	28.6	29.1
21	32.2	32.1	27.5	23	35.3	33.3	27.6
25	30.2	29.1	27.0	11	32.7	33.2	31.5
13	31.3	29.7	26.7	22	30.8	27.4	26.9
Xavg	*30.8*	*29.8 (M2/M1: 96,7%)*	*26.8*	*Xavg*	*30.8*	*29.2 (M2/M1: 94,8%)*	*27.5*
Grupa 3 / small tumour changes							
Control – K3				**Selol – S3**			
Mouse no.	**Week 1**	**Week 5**	**Week 8**	**Mouse no.**	**Week 1**	**Week 5**	**Week 8**
	M1	**M2**	**M3***		**M1**	**M2**	**M3***
12P	27.7	28.2	25.6	31	28.4	23.9	-
30	31.2	25.9	26.8	12	32.7	29.8	30.1
4	32.4	30.5	30.3	28	29.7	28.1	-
14	32.1	31.8	29.5	29	33.3	31.4	31.3
10	30.2	32.2	29.6	20	33.7	33.6	33.8
Xavg	*30.7*	*29.7 (M2/M1: 96,7%)*	*28.4*	*Xavg*	*31.6*	*29.4 (M2/M1: 93,0%)*	*31.7*
Xavg overall	**30.7**	**29.2**	**27.1**	***Xavg overall***	**31.2**	**29.2**	**28.4**

Table 3. Summary of results of changes in PSA concentrations [ng/ml] and size of isolated tumours [g], throughout the protocol. Day 1 - start - inoculation with LNCaP cells (PSA concentration < 0.003 ng/ml). Week 5 - separation into groups based on recorded PSA1 values and start of Selol / sunflower oil (placebo) dosing. Week 8 - end of protocol - isolation of blood and tumours and recording of PSA2 values.

Group 1 / large tumours							
Control – K1				**Selol – S1**			
Mouse no.	**Week 5**	**Week 8**		**Mouse no.**	**Week 5**	**Week 8**	
	PSA1	**PSA2**	**Tumour mass**		**PSA1**	**PSA2**	**Tumour mass**
5	44.2	117.3	0.7598	2	62.5	217.2	1.3652
27	26.1	84.9	0.6954	8	33.0	228.0	1.3636
3	21.0	85.1	0.3980	18	23.1	14.3	0.0482
26	20.3	68.9	0.6158	19	20.7	60.4	0.4588
6	18.5	43.7	0.2768	24	Not recorded	67.1	0.3437
Xavg	*26.0*	*80.0*	*0.5492*	*Xavg*	*34.8*	*117.4*	*0.7159*
Group 2 / medium tumours							
Control – K2				**Selol – S2**			
Mouse no.	**Week 5**	**Week 8**		**Mouse no.**	**Week 5**	**Week 8**	
	PSA1	**PSA2**	**Tumour mass**		**PSA1**	**PSA2**	**Tumour mass**
16	11.5	47.0	0.3731	1	13.9	46.5	0.2275
9	11.2	25.8	0.2621	7	11.7	65.8	0.4062
21	12.9	41.4	0.2571	23	15.8	60.4	0.5865
25	9.1	69.5	0.6035	11	11.5	71.9	0.3463
13	7.1	41.0	0.3450	22	8.6	45.8	0.1656
Xavg	*10.4*	*44.9*	*0.3682*	*Xavg*	*12.3*	*58.1*	*0.3464*
Group 3 / small tumour changes							
Control – K3				**Selol – S3**			
Mouse no.	**Week 5**	**Week 8**		**Mouse no.**	**Week 5**	**Week 8**	
	PSA1	**PSA2**	**Tumour mass**		**PSA1**	**PSA2**	**Tumour mass**
12P	4.7	30.5	0.1563	31*	6.2	-	-
30	2.4	10.1	0.0680	12	3.7	51.3	0.2406
4	2.2	5.8	0.0442	28*	2.1	-	-
14	1.5	17.7	0.1285	29	1.9	4.1	0.0195
10	0.1	4.8	0.0355	20	0.5	1.5	No tumour
Xavg	*2.2*	*13.8*	*0.0865*	*Xavg*	*2.9*	*19.0*	*0.1301*

** mouse died in week 7 of the protocol*

Table 4. C_T values for genes chosen for normalisation of gene expression data for cells isolated from tumours induced in mice using LNCaP cells.

Gene Symbol	**Sunflower oil (placebo)**				**Selol 17 mg/kg b.m./day**			
	Rep1	Rep2	Rep3	Rep1	Rep1	Rep2	Rep3	Rep1
B2M	17.06	16.80	17.62	17.72	16.66	17.06	16.92	16.53
HPRT1	19.58	19.10	20.17	20.56	19.07	19.89	19.75	18.90
RPL13A	17.10	17.14	17.61	17.44	16.55	16.77	17.02	16.42
GAPDH	23.56	23.06	23.22	23.24	22.90	23.14	23.07	22.24
ACTB	16.02	15.95	16.48	16.25	15.80	15.90	15.97	15.58
Arithmetic Mean	18.89	18.41	19.02	19.04	18.20	18.55	18.55	17.93
Avg. of Arithmetic Mean	**18.78**				**18.31**			

Table 5. Comparison of changes in gene expression in cells isolated from tumours induced in mice, treated with Selol or with placebo (sunflower oil).

Gene symbol	Gene name	Control group (placebo)		Test group (Selol 17 mg/kg b.m./day)		Fold change
		Avg. C_T	%RSD (n=4)	Avg. C_T	%RSD (n=4)	
ALB	Albumin	30,42	*4,75*	30,85	*4,23*	− 1.88
ALOX12	Arachidonate 12-lipoxygenase	28,96	*1,05*	28,70	*0,38*	− 1.17
ANGPTL7	Angiopoietin-like 7	33,83	*2,08*	32,57	*1,93*	+ 1.72
AOX1	Aldehyde oxidase 1	34,75	*2,93*	34,27	*2,12*	− 1.17
APOE	Apolipoprotein E	20,40	*1,74*	19,89	*2,20*	+1.02
ATOX1	ATX1 antioxidant protein 1 homolog (yeast)	18,87	*1,66*	18,22	*2,24*	+1.13
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	21,56	*2,61*	20,91	*1,29*	+1.13
CAT	Catalase	30,63	*0,75*	30,70	*1,37*	− 1.47
CCL5	Chemokine (C-C motif) ligand 5	23,28	*1,90*	23,06	*2,14*	− 1.19
CCS	Copper chaperone for superoxide dismutase	25,43	*4,80*	25,33	*2,64*	− 1.30
CSDE1	Cold shock domain containing E1, RNA-binding	32,15	*4,30*	31,53	*2,29*	+1.10
CYBA	Cytochrome b-245, alpha polypeptide	20,48	*1,73*	19,98	*2,62*	+1.01
CYGB	Cytoglobin	27,78	*2,88*	27,03	*2,51*	+1.21
DGKK	Diacylglycerol kinase, kappa	29,89	*1,82*	29,57	*0,95*	− 1.11
DHCR24	24-dehydrocholesterol reductase	22,56	*1,01*	22,26	*1,91*	− 1.13
DUOX1	Dual oxidase 1	23,25	*0,68*	22,82	*1,77*	− 1.03
DUOX2	Dual oxidase 2	28,88	*2,62*	28,55	*2,08*	− 1.11
DUSP1	Dual specificity phosphatase 1	23,19	*2,06*	22,48	*2,38*	+ 1.18
EPHX2	Epoxide hydrolase 2, cytoplasmic	17,31	*0,61*	16,80	*0,84*	+ 1.02
EPX	Eosinophil peroxidase	25,44	*1,71*	24,58	*2,41*	+ 1.30
FOXM1	Forkhead box M1	23,96	*2,05*	23,33	*2,48*	+ 1.12
GLRX2	Glutaredoxin 2	19,15	*1,77*	18,89	*1,66*	− 1.16
GPR156	G protein-coupled receptor 156	28,55	*0,92*	28,19	*0,23*	− 1.09
GPX1	Glutathione peroxidase 1	29,63	*1,32*	29,74	*2,24*	− 1.50
GPX2	Glutathione peroxidase 2 (gastrointestinal)	19,42	*1,17*	19,12	*1,75*	− 1.13
GPX3	Glutathione peroxidase 3 (plasma)	34,53	*2,75*	35,00	*0,00*	− 1.93
GPX4	Glutathione peroxidase 4 (phospholipid hydroperoxidase)	35,00	*0,00*	35,00	*0,00*	− 1.39
GPX5	Glutathione peroxidase 5 (epididymal androgen-related protein)	33,90	*2,80*	34,18	*2,88*	− 1.69
GPX6	Glutathione peroxidase 6 (olfactory)	25,38	*2,16*	24,74	*2,11*	+ 1.12
GPX7	Glutathione peroxidase 7	21,73	*1,81*	21,19	*2,43*	+ 1.04
GSR	Glutathione reductase	29,78	*2,80*	29,39	*1,92*	− 1.06
GSS	Glutathione synthetase	22,80	*0,63*	22,60	*2,45*	− 1.21
GSTZ1	Glutathione transferase zeta 1	21,20	*1,88*	20,72	*1,58*	+ 1.00
GTF2I	General transcription factor II, i	23,69	*2,38*	22,57	*1,56*	+ 1.56
KRT1	Keratin 1	29,37	*1,65*	29,50	*1,48*	− 1.52
LPO	Lactoperoxidase	35,00	*0,00*	35,00	*0,00*	− 1.39

MBL2	Mannose-binding lectin (protein C) 2, soluble (opsonic defect)	34,78	*0,81*	33,89	*2,05*	+ 1.33
MGST3	Microsomal glutathione S-transferase 3	22,61	*1,93*	22,10	*1,51*	+ 1.02
MPO	Myeloperoxidase	34,78	*0,88*	34,81	*1,08*	− 1.43
MPV17	MpV17 mitochondrial inner membrane protein	21,79	*2,36*	21,11	*2,45*	+ 1.15
MSRA	Methionine sulfoxide reductase A	34,87	*0,75*	35,00	*0,00*	− 1.52
MT3	Metallothionein 3	22,69	*2,00*	22,23	*1,93*	− 1.01
MTL5	Metallothionein-like 5, testis-specific (tesmin)	27,47	*1,20*	26,92	*1,32*	+ 1.06
NCF1	Neutrophil cytosolic factor 1	33,33	*1,73*	33,24	*1,70*	− 1.31
NCF2	Neutrophil cytosolic factor 2	26,98	*2,52*	26,72	*1,63*	− 1.16
NME5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	28,90	*2,08*	29,65	*0,94*	**− 2.34**
NOS2	Nitric oxide synthase 2, inducible	31,34	*3,72*	30,92	*2,39*	− 1.04
NOX5	NADPH oxidase, EF-hand calcium binding domain 5	34,29	*2,82*	34,62	*2,22*	− 1.75
NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	25,91	*1,01*	25,51	*1,65*	− 1.05
OXR1	Oxidation resistance 1	22,64	*1,87*	21,87	*2,41*	+ 1.23
OXSR1	Oxidative-stress responsive 1	24,74	*1,84*	24,09	*2,29*	+ 1.13
PDLIM1	PDZ and LIM domain 1	23,62	*2,03*	23,15	*1,82*	− 1.00
IPCEF1	Interaction protein for cytohesin exchange factors 1	23,39	*1,30*	22,96	*2,06*	− 1.04
PNKP	Polynucleotide kinase 3'-phosphatase	24,58	*2,93*	23,51	*1,82*	+ 1.51
PRDX1	Peroxiredoxin 1	24,30	*1,44*	24,15	*1,75*	− 1.25
PRDX2	Peroxiredoxin 2	22,88	*1,99*	22,35	*2,30*	+ 1.04
PRDX3	Peroxiredoxin 3	19,55	*1,37*	19,41	*2,64*	− 1.27
PRDX4	Peroxiredoxin 4	20,06	*2,85*	19,38	*4,22*	+ 1.16
PRDX5	Peroxiredoxin 5	20,92	*2,89*	20,40	*2,41*	+ 1.03
PRDX6	Peroxiredoxin 6	21,05	*0,98*	20,52	*1,94*	+ 1.04
PREX1	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	20,31	*2,16*	19,70	*2,17*	+ 1.09
PRG3	Proteoglycan 3	32,14	*2,93*	31,53	*3,24*	+ 1.10
PRNP	Prion protein	22,63	*2,90*	22,04	*2,51*	+ 1.08
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	34,24	*3,53*	31,44	*2,68*	+ 1.72
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	33,19	*4,55*	33,42	*4,05*	− 1.62
PXDN	Peroxidasin homolog (Drosophila)	23,62	*0,29*	23,24	*2,91*	− 1.07
PXDNL	Peroxidasin homolog (Drosophila)-like	22,33	*2,18*	21,70	*1,84*	+ 1.11
RNF7	Ring finger protein 7	30,58	*2,62*	29,85	*3,62*	+ 1.19
SCARA3	Scavenger receptor class A, member 3	23,13	*3,44*	22,58	*4,98*	+ 1.05
SELS	Selenoprotein S	27,56	*2,54*	27,85	*2,91*	− 1.70
SEPP1	Selenoprotein P, plasma, 1	31,56	*3,41*	30,85	*2,36*	+ 1.17
SFTPD	Surfactant protein D	23,77	*3,00*	23,57	*2,41*	− 1.21
SGK2	Serum/glucocorticoid regulated kinase 2	20,38	*2,01*	19,79	*1,59*	+ 1.08
SIRT2	Sirtuin (silent mating type information	23,19	*2,57*	22,77	*1,51*	− 1.04

	regulation 2 homolog) 2 (S. cerevisiae)					
SOD1	Superoxide dismutase 1, soluble	24,58	*2,85*	24,08	*1,42*	+ 1.01
SOD2	Superoxide dismutase 2, mitochondrial	19,78	*1,44*	19,22	*1,36*	+ 1.06
SOD3	Superoxide dismutase 3, extracellular	23,38	*2,01*	22,65	*1,70*	+ 1.19
SRXN1	Sulfiredoxin 1 homolog (S. cerevisiae)	23,07	*1,30*	22,61	*0,96*	− 1.01
STK25	Serine/threonine kinase 25 (STE20 homolog, yeast)	28,64	*1,71*	28,94	*0,79*	− 1.71
TPO	Thyroid peroxidase	31,83	*4,74*	30,40	*6,51*	+ 1.94
TTN	Titin	20,33	*2,60*	19,67	*2,54*	+ 1.14
TXNDC2	Thioredoxin domain containing 2 (spermatozoa)	22,13	*2,27*	21,47	*2,88*	+ 1.14
TXNRD1	Thioredoxin reductase 1	24,65	*3,04*	24,36	*1,96*	− 1.14
TXNRD2	Thioredoxin reductase 2	22,80	*3,48*	22,57	*3,48*	− 1.19

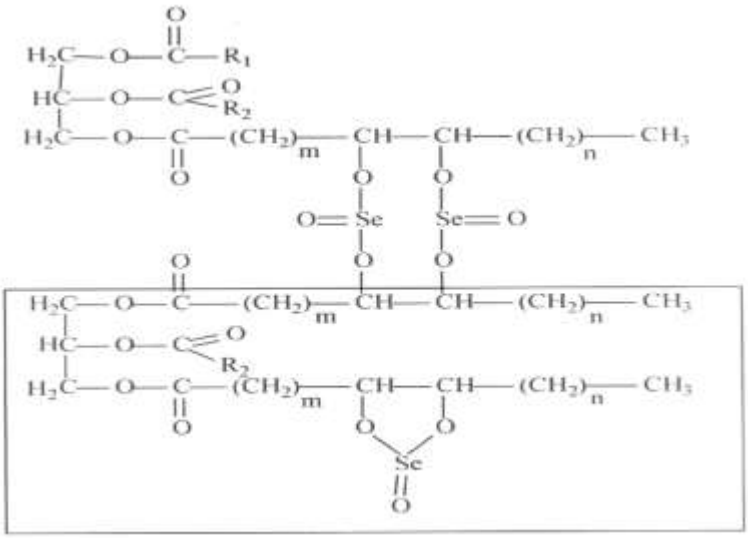

Figure 1. Probable structure of Selol containing 5% Se (IV) built into fatty acid chains of sunflower oil [13]

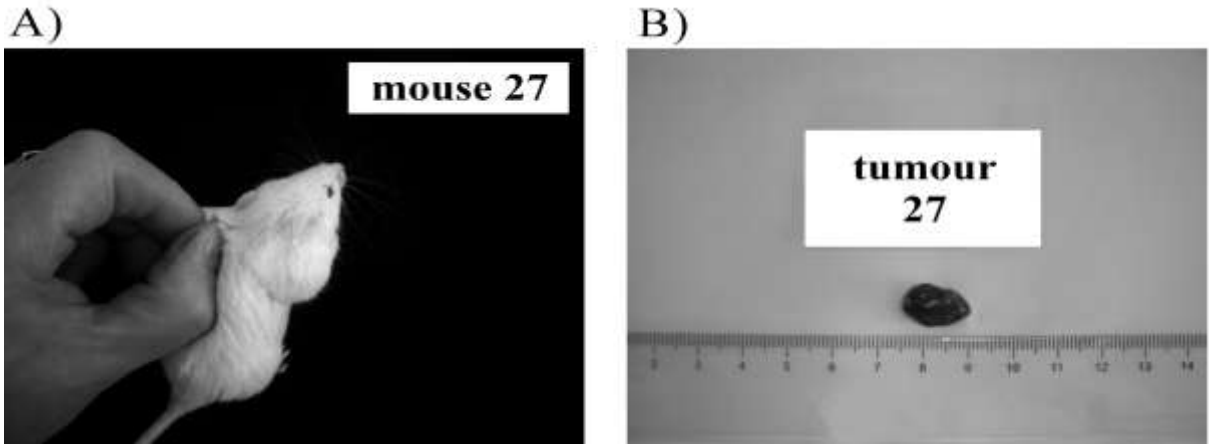

Figure 2. Mouse after 8 weeks following inoculation with LNCaP cells, with developed tumour A) and tumour following isolation B)

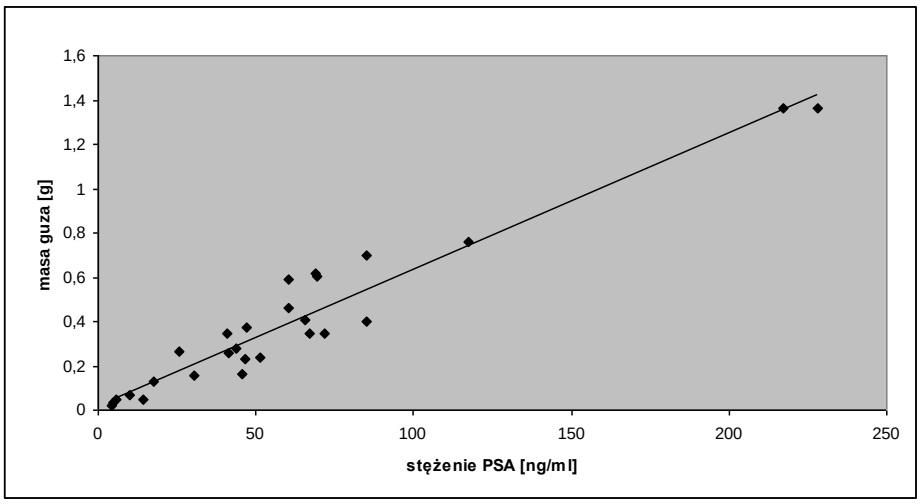

Figure 3. Relation between tumour mass and mouse blood plasma PSA concentration

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