Bioorganic & Medicinal Chemistry 18 (2010) 1783–1788

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Exploring a synthetic organoselenium compound for antioxidant pharmacotherapy—toxicity and effects on ROS-production

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ARTICLE INFO

Article history:
Received 18 November 2009
Revised 21 January 2010
Accepted 22 January 2010
Available online 28 January 2010

Keywords:
Antioxidant
Organoselenium compound
Toxicity
ROS
Neutrophil
Macrophage
ABTS

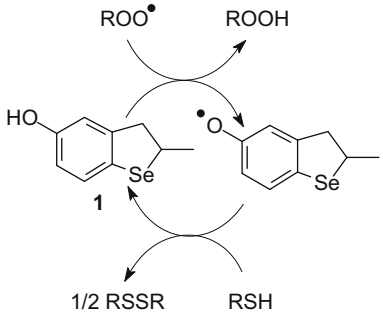
ABSTRACT

The organoselenium antioxidant **1** was previously found to act as a catalytic antioxidant in a two-phase lipid peroxidation system. In aqueous environment, selenide **1** quenched ABTS-radicals more efficiently than Trolox and ascorbic acid. The selenide dose-dependently scavenged reactive oxygen and nitrogen species more efficiently than Trolox for neutrophils and PMA-stimulated macrophages, with 50% inhibitory concentrations in the low micromolar range. In addition no sign of toxicity or effect on cell viability was seen when culturing five human cell lines in concentrations up to 200 μM of selenide **1** for up to seven days. We therefore feel that the compound would be a good candidate for future drug development for prevention or treatment of disorders caused by or involving free radical-mediated or oxidative tissue damage.

1. Introduction

Chain-breaking antioxidants in nature[1] and in man-made materials[2] often contain phenolic or aromatic amine functional groups. Due to resonance stabilization of the resulting phenoxyl or aminyl radicals, transfer of a hydrogen atom from these species to peroxyl radicals occurs much faster than the chain-propagating step of autoxidation. This transformation of a reactive peroxyl radical into a relatively harmless O- or N-centered radical is the basis for the chain-breaking antioxidant activity of phenols and aromatic amines. α-Tocopherol is the most prominent chain-breaking antioxidant in man. Just like many other phenolic antioxidants, it is known to trap two peroxyl radicals before it is converted into non-radical products. However, it would seem wiser, more sustainable and more atom-economic for nature to see that this efficient antioxidant molecule could be regenerated and used over and over again. Indeed, this catalytic process seems to go on in biological membranes where α-tocopherol is the only antioxidant present. It is thought[3] that ascorbate serves as the stoichiometric reducing agent, transferring a hydrogen atom to the α-tocopheroxyl radical at the lipid-aqueous interphase. It should however be emphasized that this recycling is rarely seen in other, out of the membrane, applications of α-tocopherol. In our search for simple, synthetic, chain-breaking antioxidants which could perform in a catalytic fashion in the presence of suitable stoichiometric reducing agents, we found that 2-methyl-2,3-dihydrobenzo[*b*]selenophene-5-ol (**1**, Scheme 1), was regenerated by water soluble thiols RSH (such as *N*-acetylcysteine), when assayed in a two-phase membrane mimicking system where peroxidation of linoleic acid in chlorobenzene was stimulated by an azo-initiator.[4] Although selenide **1** did not quench lipidperoxyl radicals as efficiently as α-tocopherol, the natural antioxidant was easily outperformed when it came to duration of inhibition. The synthetic antioxidant was active until all thiol was oxidized to the corresponding disulfide in the aqueous phase.

A proposed mechanism of catalysis,[4] involving electron transfer from thiol to phenoxyl radical followed by proton transfer and dimerisation of thiyl radicals, is shown in Scheme 1.



Scheme 1. Proposed mechanism for the catalytic chain-breaking action of organoselenium **1** in a two-phase lipid peroxidation system.

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doi:10.1016/j.bmc.2010.01.057

Production of reactive oxygen species (ROS) by leukocytes and macrophages is an important response in the immune system and a potent microbicidal mechanism of host defence. However, overproduction of these metabolites can be harmful not only to the immune cells but also to surrounding tissue because of damage caused by oxidation of macromolecules such as membrane lipids, proteins, enzymes and nucleic acids. The effects of excessive ROS formation is counteracted by the various enzymatic (catalase, SOD, glutathione peroxidases) and non-enzymatic (vitamin E, ascorbate, glutathione) antioxidant systems. Under normal conditions the levels of oxidants and antioxidants are more or less in balance.

Excessive formation of ROS and reactive nitrogen species (RNS) such as superoxide, hydrogen peroxide and peroxynitrite has been implicated in a number of pathologies,[5] including allergic/inflammatory[6,7] conditions (asthma, rheumatoid arthritis, ulcerative cholitis and Crohn's diease), atherosclerosis,[8] stroke,[9] diabetes, cancer and neurodegenerative diseases[10,11] (Alzheimer's disease, Parkinson's disease). All these diseases have at least one component of oxidative stress,[12] that is, a condition characterized in a serious imbalance between production of ROS/RNS and the various natural antioxidant defences. Antioxidant pharmacotherapy in various forms[13,14] has emerged as a means to minimize the biomolecular damage caused by attack of ROS and RNS on vital constituents of living organisms. In the present work we explored the potential and possibilities of selenide **1** for antioxidant pharmacotherapy. Introduction of a new drug into the market is a long and complex process including, as the major phases, drug discovery (target selection, hit identification, lead optimization), drug development (preclinical and clinical) and commercialization.[15] An issue which is addressed early on in this work is toxicity. Selenium is an essential trace element to man. It is incorporated into 25 different selenoproteins[16]—several with a known antioxidant function and several with a function that has not yet been elucidated. On the other hand, many inorganic and organic selenium compounds have turned out to be toxic to man.[17] In the following we report on the antioxidant capacity of selenide **1** in aqueous systems, its low toxicity in five human cell-lines and its ability to efficiently scavenge ROS-production in neutrophils and stimulated macrophages.

2. Results and discussion

2.1. Antioxidant capacity in aqueous solution as determined by the ABTS-assay

Previously, the antioxidant capacity of selenide **1** has only been tested in lipid (chlorobenzene) phase.[4] In order to get a rough idea about its aqueous phase performance, we determined its Trolox Equivalent Antioxidant Capacity (TEAC),[18] which has been used to quantify the antioxidant activity of biological fluids, extracts and pure compounds. The TEAC value is defined as the concentration of Trolox which has an equivalent antioxidant potential as a 1 mM solution of the compound under investigation.[19] An 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{\cdot+}$) assay was used to determine relative TEAC values. To a preformed solution of $ABTS^{\cdot+}$ in PBS buffer, pH 7.4, with an absorbance slightly above 0.7 was added various amounts (3–30 μM) of selenide **1**, Trolox, sodium ascorbate and *N*-acetylcysteine.

The absorbance at 734 nm was recorded immediately after mixing and then every minute for the next 8 min. Experiments carried out at 6.08 μM concentrations could clearly distinguish the quenching capacity of selenide **1** and the three water-soluble reference antioxidants (Fig. 1). Selenide **1** clearly outperformed both sodium ascorbate and Trolox when it came to the number of $ABTS^{\cdot+}$-radicals quenched by each molecule of the antioxidant. The data presented in Figure 1 would correspond to a TEAC value after 5 min of 1.6 for selenide **1**. Similarly to sodium ascorbate and Trolox, selenide **1** quenched $ABTS^{\cdot+}$-radicals promptly and the oxidation products formed continued to quench radicals only at a very slow rate. With *N*-acetylcysteine, which showed a higher TEAC value of 2.4, the contribution from the primary oxidation products to further react with $ABTS^{\cdot+}$-radicals was much larger.[20] It is clear from this part of the study that selenide **1** has a high capacity to quench radicals also in an aqueous environment.

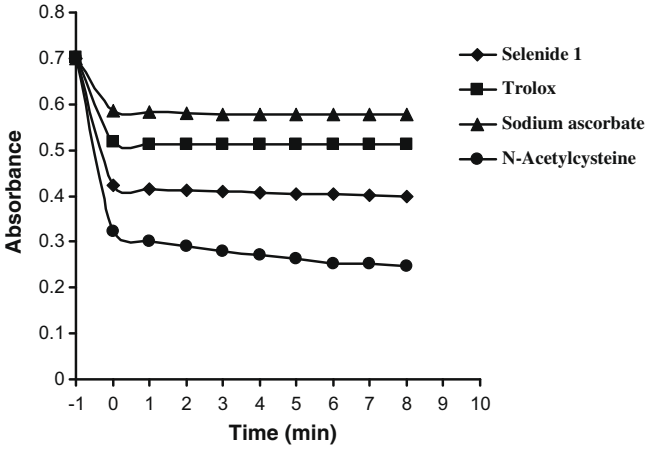

Figure 1. Absorbance at 734 nm of the $ABTS^{\cdot+}$-radical cation (47.4 μM) with time after addition of selenide **1**, Trolox, sodium ascorbate and *N*-acetylcysteine (each 6.08 μM) in aqueous PBS-buffer.

2.2. Assessment of cell viability with the Alamar Blue and LDH assays

Alamar Blue is a non-toxic metabolic indicator for viable cells. Following uptake, the dye becomes reduced and changes color. The extent of this conversion is a reflection of cell viability and correlates well with the number of living cells in the sample. As can be seen in Figure 2, the Alamar Blue assay showed normal cell growth patterns with cell numbers increasing from day 1 to day 7 both in the presence of selenide **1** (60 μM) and Trolox (60 μM) which was used as an antioxidant reference. This pattern was observed more or less independently of cell type or antioxidant additive. The broad selection of human cell types in addition to the relatively high concentration of antioxidants clearly indicates that normal cellular proliferation is not affected. Considerably higher concentrations of selenide **1** were also tested on these cell-lines. These experiments indicated no sign of cell death or decreased proliferation until concentrations as high as 200 μM (data not shown).

Cell death and plasma membrane damage was also assayed by measuring the release of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in most cells. In the kit provided by Sigma, LDH participates in a reaction which converts a yellow tetrazolium salt into a red formazan-class dye. The amount of formazan is directly proportional to the amount of LDH in the culture, which in turn is proportional to the number of dead or damaged cells. The LDH assay correlated well with the proliferation data, that is, no significant signs of toxicity for any of the cell types could be seen as a consequence of addition of selenide **1** (60 μM) or Trolox (60 μM) (Fig. 3).

2.3. Effects of selenide 1 on free radical scavenging as determined by ROS production by neutrophils and macrophages

To evaluate the antioxidant activity of selenide **1** in a more biological setting, we investigated the capacity of the material to quench ROS produced by freshly isolated human neutrophils and PMA (Phorbol Myristate Acetate)-stimulated THP-1 cells. The antioxidant capacity of selenide **1** was again compared to those

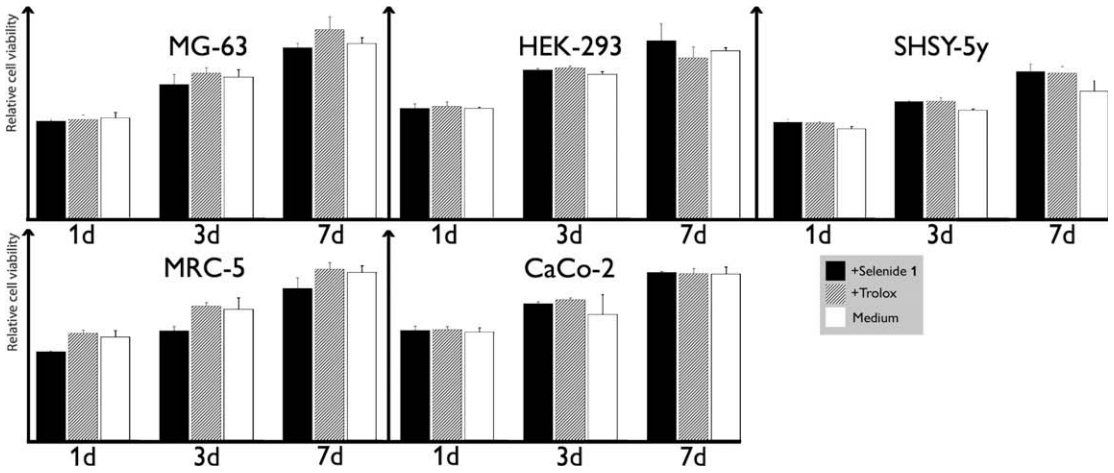

Figure 2. Relative viability of MG-63, HEK-293, SHSY-5y, MRC-5 and CaCo-2-cells in the presence/absence of 60 μM of selenide **1** or Trolox as determined by Alamar Blue measurements (absorbance, 570 nm) after 1 day (1 d), 3 days (3 d), and 7 days (7 d).

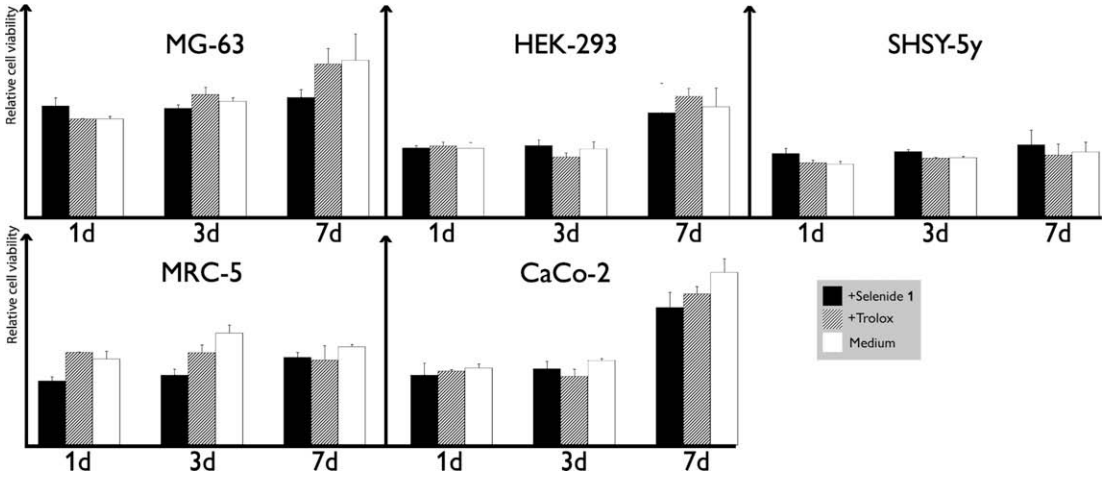

Figure 3. Relative cytotoxicity caused by 60 μM of selenide **1** or Trolox as determined by LDH measurements (absorbance, 570 nm), after 1 day (1d), 3 days (3d), and 7 days (7d) of incubation with MG-63, HEK-293, SHSY-5y, MRC-5 and CaCO-2-cells.

of Trolox, a water soluble derivative of vitamin E, which is commonly used in biological and biochemical applications to reduce oxidative stress or damage. Total ROS (i.e., both extra- and intra-cellular production) was measured using modified luminol enhanced chemiluminescence (CL).[21] As can be seen in Figures 4 and 6 (neutrophils and macrophages, respectively), selenide **1** showed a clear, dose dependent, quenching/inhibiting effect on ROS production, independent of cell source. Experiments with selenide **1** in concentrations ranging from 0.1 to 120 μM indicated a 50% inhibitory concentration of 1 μM for inhibition of ROS in neutrophils (data not shown). Figures 5 and 7 demonstrate that selenide **1** is considerably more effective than Trolox in quenching ROS produced both from neutrophils and THP-1 cells. Thus, we have demonstrated antioxidant capacity of selenide **1** both during short term (neutrophils) and long-term (macrophages) production of ROS.

Selenoureas and selenoamides were recently found to scavenge ROS-production in polymorphonuclear leukocytes with 50% inhibitory concentrations in the range of 7 μM.[22] The antioxidative effect did not seem to be due to inhibition of NADPH oxidase and it was hypothesized that the organoselenium compounds could scavenge superoxide directly to produce oxygen. Selenide **1** is structurally very different from the selenoureas and selenoamides and the reason for its ROS-inhibiting activity still remains to be elucidated.

3. Conclusions

In aqueous environment, the phenolic organoselenium antioxidant 2-methyl-2,3-dihydrobenzo[*b*]selenophene-5-ol (**1**) quenched ABTS-radicals more efficiently than Trolox and ascorbic acid. Previously, the high antioxidant capacity of the organoselenium had only been documented in lipid phase. The selenide dose-dependently scavenged reactive oxygen- and nitrogen species more efficiently than Trolox when using neutrophils and PMA-stimulated macrophages. Furthermore, no sign of toxicity or effect on cell viability was seen in five human cell lines for concentrations up to 200 μM and seven days of exposure. Thus, the compound would be a good candidate for future drug development, specifically for prevention or treatment of disorders caused by or involving free radical-mediated or oxidative tissue damage.

4. Experimental

4.1. Materials

2-Methyl-2,3-dihydrobenzo[*b*]selenophene-5-ol (**1**) was prepared as described in the literature.[4]

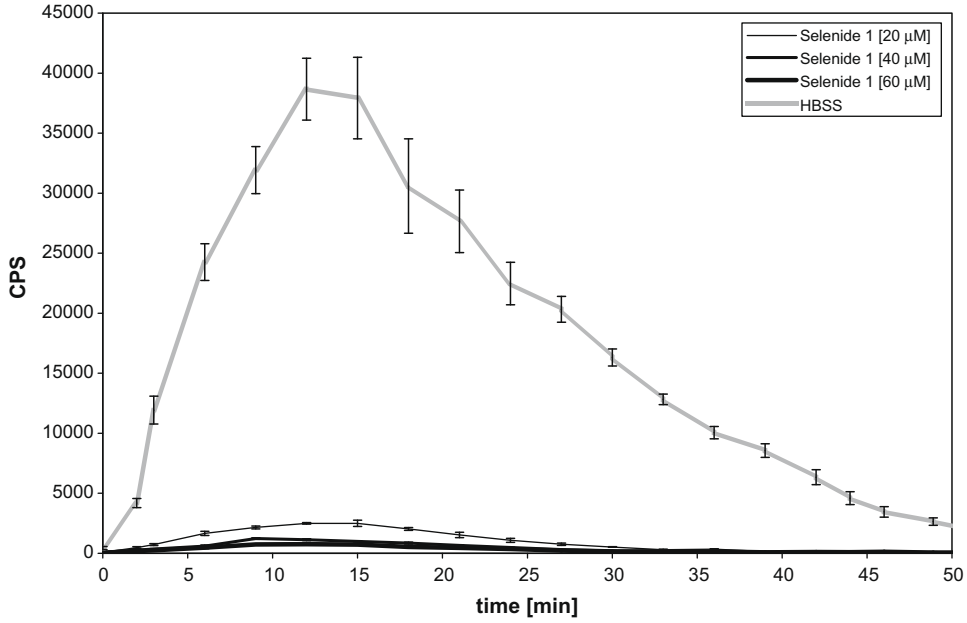

Figure 4. Chemiluminescence measured in counts per second (CPS) for human neutrophils exposed to various concentrations of selenide **1** (20, 40, 60 μM) in comparison with control (HBSS).

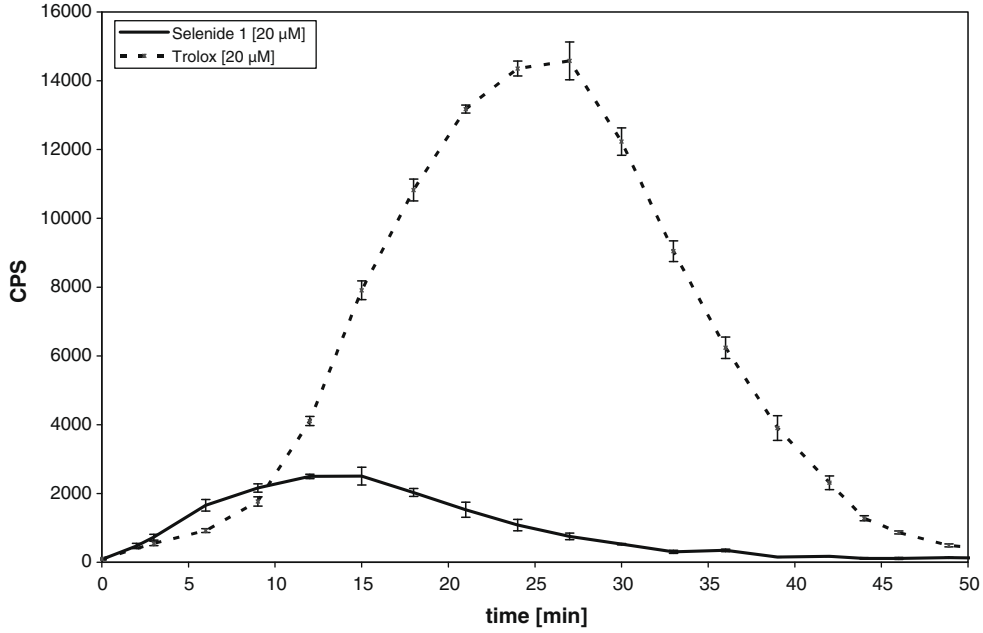

Figure 5. Chemiluminescence measured in counts per second (CPS) for human neutrophils exposed to selenide **1** (20 μM) or Trolox (20 μM).

4.2. ABTS-assay

Antioxidant activity as determined by the capacity of the antioxidant to decolorize the $ABTS^{\cdot+}$ radical cation was determined essentially as described in the literature.[19] Briefly, ABTS, (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt; 5.76 mg, 0.0105 mmol), was allowed to react with less than the stoichiometric amount of potassium persulfate (1.00 mg, 0.0037 mmol) in water (1.5 mL) to produce, after 15 h, a stock solution of the corresponding radical cation with a stable absorbance. This stock solution (0.0200 mL), diluted with PBS buffer—pH 7.4 (1.48 mL), showed an absorbance of 0.711 at 734 nm. Using an extinction coefficient of 1.5×10^4 for the radical cation $ABTS^{\cdot+}$ in water, this value corresponds to a 47.4 μM concentration of the $ABTS^{\cdot+}$ radical cation. In a typical experiment, the antioxidant (1.52 μL of a 3.00 mM solution in MeOH or water; final concentration = 6.08 μM) was syringed into 0.750 mL of the $ABTS^{\cdot+}$ radical cation solution. After initial mixing, the absorbance was recorded every minute for the next 8 min. Figure 1 shows a plot of absorbance versus time for experiments carried out with Trolox, organoselenium compound **1**, sodium ascorbate and *N*-acetylcysteine.

4.3. Cell culture

MG-63, SHSY-5Y, MRC-5, Caco-2 and HEK 293 were cultured in DMEM:F12 (Sigma–Aldrich) supplemented with 10% FCS, 100 IU

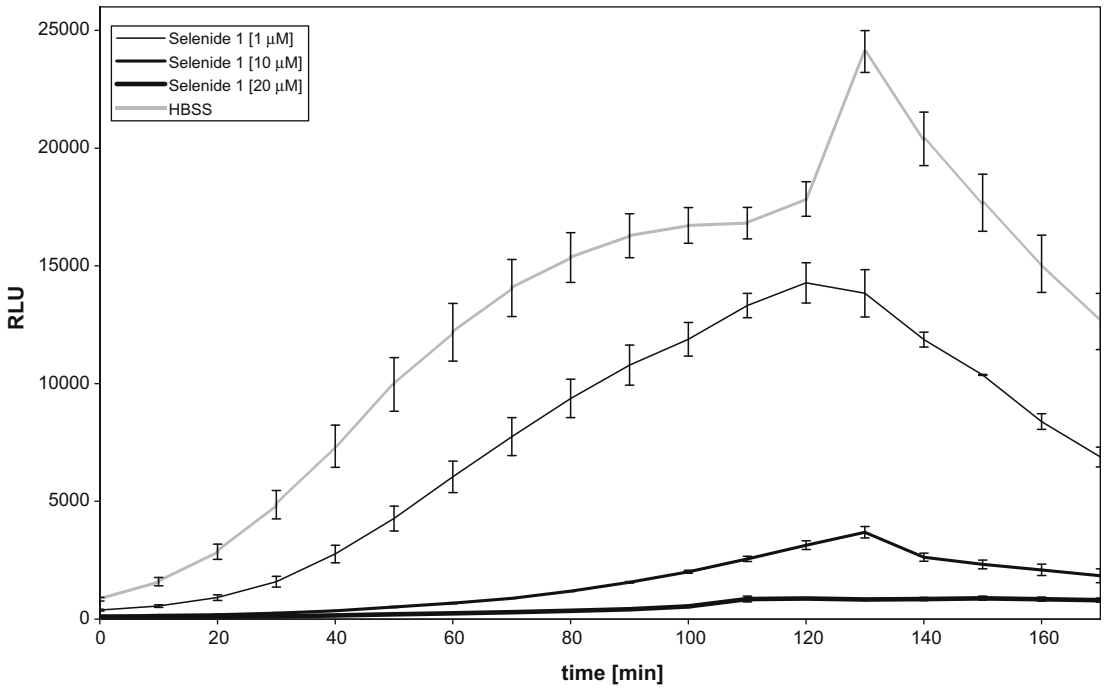

Figure 6. Chemiluminescence measured in relative light units (RLU) for PMA stimulated THP-1 cells exposed to selenide **1** (1, 10 and 20 μM) in comparison with control (HBSS).

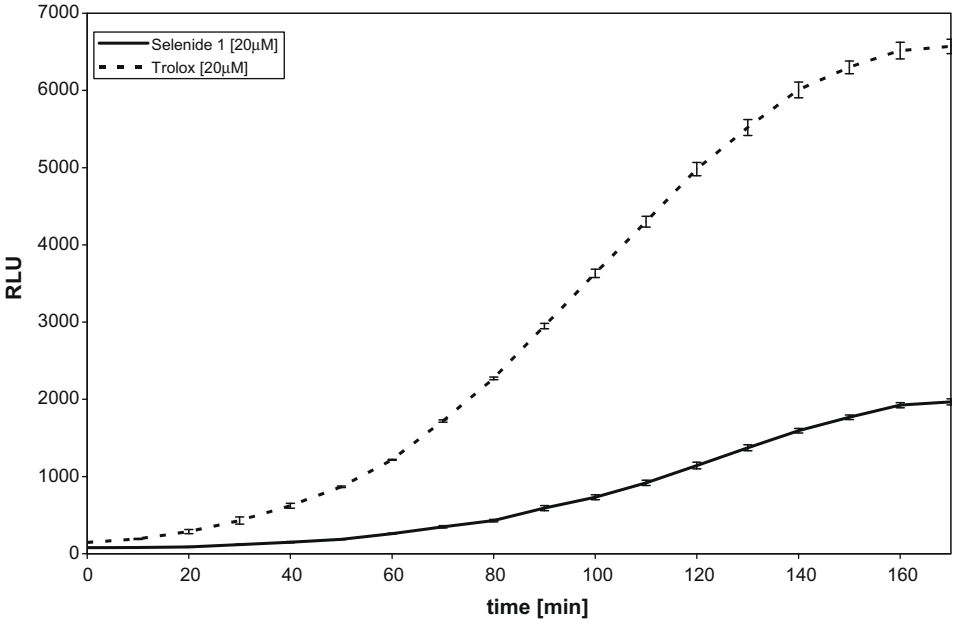

Figure 7. Chemiluminescence measured in relative light units (RLU) for PMA stimulated THP-1 cells exposed to selenide **1** (20 μM) or Trolox (20 μM).

penicillin/mL, 100 μg streptomycin/mL, 2 nM L-glutamine and 1% non-essential amino acids at 37 °C, 5% CO_2 in a humidified atmosphere.

The THP-1 cells were cultured in RPMI-1640 (GIBCO®) supplemented with 10% FCS, 100 IU penicillin/mL, 100 μg streptomycin/mL, 2 nM L-glutamin and 1% non-essential amino acids at 37 °C, 5% CO_2 in a humidified atmosphere.

4.4. Neutrophil isolation

Neutrophils were isolated from heparinized blood of apparently healthy blood donors (Academic Hospital, Uppsala, Sweden) following a routine dextran sedimentation method essentially as described by Håkansson and Venge.[23] To each of three test tubes containing 5 mL heparinized blood, dextran (T500, Pharmacia, Uppsala, Sweden) was added (final concentration 1%) and the sedimentation was allowed to proceed for 30 min at room temperature. The neutrophil rich supernatant was harvested, pooled and centrifuged at 160 g for 5 min. The pellet was washed twice with 0.9% NaCl. Contaminating erythrocytes were lysed by a 30 s exposure to water (Milli Q) after which 3.6% NaCl was added to reach a final concentration of 0.9%. The obtained suspension was then centrifuged for 5 min at 160 g. Finally, the pellet was resuspended in Gey's buffer and the average content of neutrophils (approximately

90%) was calculated using Türks staining and hemocytometer method. The experiments were performed within 1–2 h after neutrophil isolation.

4.5. Cell proliferation and cytotoxicity assays

MG-63, SHSY-5Y, MRC-5, Caco-2 and HEK 293 were harvested using trypsin-EDTA treatment. The cells were centrifuged at 400*g* for 5 min after which they were resuspended in phenol red free Alpha medium (GIBCO®) containing 10% FCS, 100 IU penicillin/ml, 100 μg streptomycin/ml, 2 nM L-glutamin and 1% non-essential amino acids.

30,000 cells/well (of each cell type) and 60 μM of the antioxidants Trolox or SeOH were added to a 24 well plate in triplicate samples. Cells w/o antioxidant were used as controls.

4.5.1. Alamar Blue

Culture medium was removed from the wells after 1, 3 and 7 days and replaced by 0.5 mL Alamar Blue stock solution (Serotec) diluted 1:10 in Hanks balanced salt solution (HBSS) and incubated at 37 °C, 5% CO_2 in a humidified atmosphere for 2.5 h. Alamar Blue is a non-toxic metabolic indicator for viable cells. Upon uptake into the cell the dye becomes reduced and changes color. The color change correlates approximately with the number of living cells in the sample. Aliquots of 100 μL from each well were transferred to a 96-well plate and the absorbance was read at 570 nm using a multiscan MS spectrophotometer (Labsysytems).

4.5.2. LDH

Culture medium was removed from the wells after 1, 3 and 7 days and used for cytotoxicity measurements using an LDH in vitro toxicology assay kit (Sigma®) according to the manufacturer's protocol.

Cell death and plasma membrane damage can be assayed measuring the release of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in most cells. LDH participates in a coupled reaction, which converts a yellow tetrazolium salt into a red, formazan-class dye. The amount of formazan is directly proportional to the amount of LDH in the culture, which is in turn proportional to the number of dead or damaged cells. Absorbance was read at 570 nm using a multiscan MS spectrophotometer (Labsystems).

4.6. Chemiluminescence (CL)

4.6.1. Neutrophils

The generation of oxygen free radicals over time from neutrophils was monitored for 50 min in 24 well white optiplates (Greiner) using a Wallac Victor in the luminescence mode.

Approximately 300,000 neutrophils diluted in HBSS containing different concentrations of selenide **1** and Trolox (20, 40 or 60 μM) was added per well to a 24 well plate together with 100 μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), 0.17 M NaOH and 6,4 U/mL horseradish peroxidase (HRP).[21] Luminescence intensity was read every 5 min. The measurements were commonly performed on quadruplicate samples.

4.6.2. THP-1

The generation of oxygen free radicals over time from macrophages (THP-1) was monitored for 180 min in 96-well white optiplates (Greiner) using a using a TECAN reader and Diogenes chemiluminescence kit (National Diagnostics).

Approximately 200,000 THP-1 cells diluted in HBSS containing different concentrations of selenide **1** and Trolox (20, 40 or 60 μM) was added per well to a 96 well plate together with Diogenes reagent 20 v/% (following the manufacturers recommendations) and 40 μM Phorbol Myristate Acetate (PMA). Luminescence intensity was read every 6 min. The measurements were commonly performed in quadruplicate.

4.7. Statistical analysis

For the in vitro assays, the mean values and standard deviations are based on triplicate or quadruplicate samples. The experiments have been repeated at least twice with similar results.

Acknowledgements

We thank Professors Staffan Johansson and Kathrin Zeller at the department of Medical Biochemistry and Microbiology, Uppsala University, for kindly providing THP-1 cells and Professors Fredrik Palm and Angelica Fasching at the department of Medical Cell Biology, Uppsala University for the use of the TECAN luminometer. The Swedish Research Council is gratefully acknowledged for financial support.

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