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Reaction kinetics and targeting to cellular glutathione S-transferase of the glutathione peroxidase mimetic PhSeZnCl and its D,L -polylactide microparticle formulation

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Abstract

Catalytic properties and cellular effects of the glutathione peroxidase (GPx)-mimetic compound PhSeZnCl or its D,L -lactide polymer microencapsulation form (M-PhSeZnCl) were investigated and compared with the prototypical Se-organic compounds ebselen and diselenide (PhSe)₂. PhSeZnCl was confirmed to catalyze the ping-pong reaction of GPx with higher V_{max} than ebselen and (PhSe)₂, but the catalytic efficiency calculated for the cosubstrates glutathione (GSH) and H₂O₂, and particularly the high reactivity against thiols (lowest K_M for GSH in the series of test molecules), suggested poor biological applicability of PhSeZnCl as a GPx mimetic. Cytotoxicity of PhSeZnCl was demonstrated in various cancer cell lines via increased reactive oxygen species (ROS) generation, depletion of intracellular thiols, and induction of apoptosis. Experiments carried out in GSH S-transferase P (GSTP)-overexpressing K562 human erythroleukemia cells and in GSTP1-1-knockout murine embryonic fibroblasts (MEFs) demonstrated that this cytosolic enzyme represents a preferential target of the redox disturbances produced by this Se-compound with a key role in controlling H₂O₂ generation and the perturbation of stress/survival kinase signaling. Microencapsulation was adopted as a strategy to control the thiol reactivity and oxidative stress effects of PhSeZnCl, then assessing applications alternative to anticancer. The uptake of this “depowered” GPx-mimetic formulation, which occurred through an endocytosis-like mechanism, resulted in a marked reduction of cytotoxicity. In MCF-7 cells transfected with different allelic variants of GSTP, M-PhSeZnCl lowered the burst of cellular ROS induced by the exposure to extracellular H₂O₂, and the extent of this effect changed between the GSTP variants. Microencapsulation is a straightforward strategy to mitigate the toxicity of thiol-reactive Se-

organic drugs that enhanced the antioxidant and cellular protective effects of PhSeZnCl. A mechanistic linkage of these effects with the expression pattern and signaling properties of GSTP. This has overcome the GPx-mimetic paradigm proposed for Se-organic drugs with a more pragmatic concept of GSTP signaling modulators.

Keywords

Selenium; Seleno-organic compound; Antioxidants; Thiols; Glutathione peroxidase; Ebselen; Diphenyl diselenide; PhSeZnCl; Glutathione *S*-transferase Pi; Peroxiredoxins; Cell signaling

Introduction

Since the discovery of the pivotal role of selenium in the catalytic mechanism of some glutathione peroxidase (GPx; EC 1.11.1.9) isoenzymes, namely GPx's 1–4 and 6 reviewed in [1], the development of synthetic chalcogen-based catalytic antioxidants has attracted considerable attention [2–4]. Synthetic Se-organic compounds have emerged as GPx mimetic drugs recently reviewed in [5–8], owing to the redox activity of the selenium center that in the presence of two molecules of reduced glutathione (GSH) promotes the two-electron reduction of hydroperoxides (Fig. 1). The ping-pong reaction mechanism proposed for the GPx reaction process is well characterized even if biological consequences are far from being completely understood, recently reviewed in [9]. These in fact may include the redox tuning of signaling pathways by the modification of cellular peroxide flux and redox state of protein thiols.

The prototypical organo-Se drug ebselen, 2-phenyl-1,2-benzisoselenazol-3[2*H*]-one (Supplementary Table A), is a lipid-soluble cyclic selenenamide that potently inhibits lipid peroxidation through a GPx-like reaction recently reviewed in [10]. It also behaves as a potent scavenger of peroxynitrite and hydroperoxides of membrane phospholipids. This was the first synthetic drug used to inactivate H₂O₂ in clinical trials as a neuroprotective agent in acute cerebral infarction and other conditions associated with oxidative stress and defective GPx activity. Other pharmacological mechanisms proposed for this organo-Se compound include a direct inhibition of the inducible form of nitric oxide synthase [11].

Diselenides are another group of GPx-mimetic compounds that have gained consideration in previous years, reviewed in [5]. Diphenyl diselenide ((PhSe)₂) [12], the simplest structure in this series (Supplementary Table A), reacts very efficiently with hydroperoxides and organic peroxides, mimicking the reaction cycle of the GPx enzyme in the presence of reduced thiols [13]. The apparent activity of (PhSe)₂ has been reported to be higher than that of ebselen, also displaying a lower toxicity to mammalian cells [14].

Mechanistic aspects of Se-organic compounds at the cellular level include redox modulation and stress response effects recently, reviewed in [15]. These compounds stimulate to different extents the generation of reactive oxygen species (ROS), depletion of intracellular GSH, and selected modifications of the thiol/disulfide status of signaling proteins, some of which act as redox sensors to coordinate the transcriptional response to electrophilic

substances. The effects, however, are reported for some, but not all the forms of organic Se [16,17].

Phenylselenium zinc chloride (PhSeZnCl) (Fig. 1 and Supplementary Table A) is a member of the Se-organic family of compounds recently synthesized by some of us [18] as an air- and moisture-stable noncrystalline powder that, despite its lipophilic character, shows an enhanced reactivity in water. To the best of our knowledge, this is a unique example of bulk activity in which the Se atom can be easily maintained at the oxidation state -1 , the same as observed in the catalytic pocket of mammalian GPx enzymes at the beginning of the reaction cycle [19] (Fig. 1). These properties are missing in the unstable PhSeH and its oxidized derivative, the diselenide (PhSe) $_2$.

Earlier evidence demonstrated the activity of PhSeZnCl as a thiol oxidation catalyst in cell-free reaction systems [8]. In the present work we aim to further characterize this activity of PhSeZnCl in the context of the GPx catalytic mechanism. To this end, the reaction kinetics of PhSeZnCl were investigated in comparison with ebselen, (PhSe) $_2$, and its D,L-lactide polymer microencapsulation formulation (M-PhSeZnCl). Cellular effects of PhSeZnCl and M-PhSeZnCl were also investigated, assessing ROS generation, GSH levels, and the activity and signaling functions of the π isoform (P) of glutathione S-transferase (GST; EC 2.5.1.18). This enzyme contains critical Cys residues that may sense the flux of H $_2$ O $_2$ and other ROS [20], and the Se-organic drug ebselen is described to act as a potent inhibitor of its enzyme activity [21], which demonstrates the potential for this and other Se-organic molecules in chemotherapy protocols of cancers and other conditions associated with GST overexpression. GSTP-knockout (GSTP $^{-/-}$) murine embryonic fibroblasts (MEFs) have been used as a model to decipher the GST-dependent mechanism of protection and the signaling of non tumoral cells exposed to PhSeZnCl.

In this study, microencapsulation has been designed as a strategy to constrain PhSeZnCl cytotoxicity and thus to investigate GSTP-dependent and independent effects of this Se-compound as a cellular protection and redox signaling molecule. These properties of M-PhSeZnCl were assessed in MCF-7 cells transfected with allelic variants of GSTP providing different enzymatic and redox-regulating activities on peroxiredoxin VI (Prdx6), a membrane peroxidase with a proven role in preventing oxidative stress at the cellular level [22]. Here, the effects of M-PhSeZnCl on cellular ROS production upon exposure to exogenous H $_2$ O $_2$ were assessed.

Materials and methods

Se-organic compounds and microparticle formulation of PhSeZnCl

Organo-selenium compounds (Supplementary Table A) included PhSeZnCl and PhSZnCl, which were synthesized according to [18,23], and ebselen and (PhSe) $_2$, which were from Sigma-Aldrich (Milan, Italy).

A microparticle formulation of PhSeZnCl (M-PhSeZnCl) was prepared using D,L-lactide as the coating polymer (PLA R203H, MW 29000 Da, Boehringer Ingelheim, Germany). Briefly, PhSeZnCl and M-PhSeZnCl dry powders were prepared by spray-drying of acetone

solutions (Buchi mini-spray dryer Buchi-B290, Italy). Unlabeled microparticles and fluorescein isothiocyanate (FITC; Sigma–Aldrich)-labeled M-PhSeZnCl were prepared using the same method and were preliminarily tested for the effect of FITC labeling on cell uptake and viability. All preparations were characterized in terms of morphology, size distribution, and drug content. Morphological analysis was carried out by scanning electron microscopy using a field emission FEG LEO 1525 (Zeiss) microscope with a field acceleration of 10 keV. PhSeZnCl stability was assessed after spray-drying. Spectra of PhSeZnCl before and after spray-drying or loaded in M-PhSeZnCl and dissolved in d_6 -dimethyl sulfoxide were acquired by a Bruker Avance DRX-400 MHz nuclear magnetic resonance spectrometer (Bruker, Switzerland). Spray-drying reduced the size of PhSeZnCl bulk powder from 19 to 5.3 μm (Supplementary Fig. B). M-PhSeZnCl had a similar volume mean diameter of 5.5 μm and span of 1.5. M-PhSeZnCl were more regular, with a round shape and wrinkled surface, whereas dry powders had a hollow structure with rough and very irregular surface. These differences can be ascribed to the PLA wrapping that clearly entrapped PhSeZnCl particles within the polymer matrix. As a result, drug content was as high as 17% w/w and encapsulation efficiency >80%. The analysis of nuclear magnetic resonance profiles showed no particular effect on the molecule structure after spray-drying process (data not shown). Labeling with FITC did not cause any significant change of M-PhSeZnCl characteristics.

Glutathione peroxidase kinetics

Glutathione peroxidase activity of Se-compounds was studied using the glutathione reductase (GR)-coupled assay [24]. Glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*) (EC 1.8.1.7; Sigma-Aldrich) was used to reduce back the glutathione disulfide (GSSG) formed from GSH in the H_2O_2 reduction reaction of GPx, with reduced β -nicotinamide adenine dinucleotide phosphate (NADPH; Sigma-Aldrich) as a source of electrons. The molar extinction of NADPH was measured at 340 nm using an UV/Vis spectrophotometer ($\epsilon=6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and the mole equivalents of GSH consumed during the GPx catalytic cycle of H_2O_2 reduction were derived and used to calculate enzyme activity. Specific activity was expressed as international units (U), with one U corresponding to 1 μmol of GSH consumed per minute at pH 7 and 25 °C. The assay was verified using a crude GPx enzyme from bovine erythrocytes (Sigma-Aldrich; unit range in the assay mixture of 0.075–0.15). The apparent initial rate of reaction (V_0) was expressed as of absorbance/minute and was determined in the first 10 min of reading, fitting the linear region of the curve with the highest slope.

Although GPx-mimetic agents follow by definition a multisubstrate ping-pong reaction mechanism [9], the reaction kinetics of PhSeZnCl and its microencapsulated formulation M-PhSeZnCl were investigated by extrapolation to classical one-substrate Michaelis-Menten kinetics. To this end apparent rate constants were approximated to real ones, alternately keeping constant the concentration of one of the two substrates, namely GSH and H_2O_2 , and varying the other in the range 0.06–2 or 0.088–0.88 mM, respectively. Apparent Michaelis constants (K_M) and maximal rate (V_{max}) values were calculated using the standard assay procedure reported above. Data were analyzed by fitting to the Michaelis-Menten equation using a nonlinear curve-fitting program (PRISM4, GraphPad Software, Inc., San Diego, CA,

USA); K_M , V_{max} , the catalytic constant ($K_{cat}=V_{max}/[Se\text{-organic compound}]K_M$), and the catalytic coefficient ($\eta=k_{cat}K_M$) were derived by plotting the reciprocals of the initial reaction rate and of substrates. Data were the means of three independent experiments.

The apparent activity of PhSeZnCl and ebselen was also determined with a microplate assay of H_2O_2 reduction carried out with the probe Amplex red (AR). In the presence of horseradish peroxidase (HRP), the oxidation of AR forms the fluorescent product resorufin, which is measured at excitation 535 nm and emission 595 nm. Briefly, 50 μ l of sample containing the catalyst and GSH at the desired concentration was mixed in the well with 50 μ l of a detection solution containing 50 μ M AR and 10 U/ml HRP in citrate buffer 0.1 M, pH 6. The reaction was started with a bolus of H_2O_2 (1 μ M) and fluorescence kinetics were recorded for 15 min. The linear part of the reaction was used to determine initial rate values that were plotted against the concentration of the cosubstrate GSH. The assay was calibrated with authentic H_2O_2 .

Cell cultures

Immortalized MEFs were prepared from wild-type (GSTP^{+/+}) and GSTP-knockout (GSTP^{-/-}) mice and cultured as described in [25] and references therein.

K562 human erythroleukemia was maintained in RPMI 1640 containing 100 U/ml of penicillin and 100 μ g/ml streptomycin, 2 mM L-glutamine, and 10% v/v fetal bovine serum (FBS). A549 human lung adenocarcinoma epithelial (type II alveolar) cells and a human bronchial epithelial cell line (BEAS) were cultured in Dulbecco's modified Eagle's medium (Lonza) containing 10% v/v FBS, 2 mM L-glutamine, and 100 U/ml of penicillin and 100 μ g/ml streptomycin. LnCap cells were maintained in RPMI 1640 (Lonza) containing 100 U/ml of penicillin and 100 μ g/ml streptomycin, 2 mM L-glutamine, and 10% v/v FBS. MCF-7 cells, from the American Type Culture Collection (Manassas, VA, USA), were cultured in RPMI 1640 medium with 10% v/v FBS. Cell transfection was performed using the in vitro DNA transfection reagent for MCF-7 cells GenJet version II (SignaGen Laboratories, Rockville, MD, USA) with pCMV-Tag2a-Flag (Kan^R and Neo^R) empty or GSTP1-1A-encoding vectors. The site-directed mutagenesis of the GSTP1-1A vector was performed as described previously in [22] and references therein. Characteristics of the allelic variants of GSTP1-1 used in these experiments were described in [22].

All the cell cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO₂.

Cell viability and clonogenic assay protocols

Cells (0.1×10^5 cells/well) seeded in 96-well plates were incubated in medium with various concentrations of PhSeZnCl, (PhSe)₂, or ebselen for 24–48 h. Cell viability was then assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test [26] and inhibition results were reported as IC₅₀ values. Nonspecific direct oxidation of the MTT probe by the Se-compounds was observed in cell-free tests, but the conditions applied to cell tests excluded any significant interference with the results of the viability assay.

In some experiments, cell viability was also investigated with the luciferase-ATP luminescence assay kit (Promega, Madison, WI, USA). ATP levels were normalized to sample protein content.

Clonogenic assay was performed in BEAS and MCF-7 cells. These were plated in complete medium at 1×10^3 cells/well in six-well plates. Eighteen hours after plating, the medium was replaced with fresh medium with or without different doses of the Se-compound PhSeZnCl in the free or microencapsulated form. At day 9 the cells were washed in phosphate-buffered saline (PBS; $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$) and then fixed in methanol/acetic acid (3/1) for 5 min and then counted by ImageJ software after being stained with 0.5% crystal violet (in methanol) for 15 min at room temperature. The pixel of a colony consisting of at least 50 cells was considered for acquisition in the image analysis.

Apoptotic signaling

Apoptosis was investigated in K562 and MEFs treated with the Se-organic compounds PhSeZnCl and ebselen by cytofluorimetric analysis [27] using the fluorescent probes propidium iodide and annexin V-FITC.

Activity of mitogen-activated protein kinases (MAPKs) associated with cell stress and survival was investigated by immunoblotting analysis of total protein and phosphorylated forms of SAPK-JNK and ERK1/2 as described in [27,28].

Cellular ROS

Cellular ROS were assessed using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) according to [29]. The specificity of the assay for the detection of H_2O_2 and the effect of thiol depletion on cellular stress parameters were verified by cell incubation with PEG-catalase (PEG-CAT; 50 U/ml) and *N*-acetylcysteine (NAC; 10 mM), respectively. NAC or PEG-CAT was administered to cell cultures at the beginning of treatments, together with PhSeZnCl (10 or 100 μM), that lasted for 6 or 24 h. In some experiments the antioxidants were administered to cell cultures at the end of the treatment with Se-organic compound together with the DCFH-DA probe, according to [30], or 2 h before the incubation of cells with the probe. These two procedures, however, produced much lower effects on DCF fluorescence, cell viability, and thiols than the previous protocol and consequently were not included in the experimental protocol.

In MCF-7 cells transfected with GSTP1 variants mounted in plastic (Aclar) slides [22] and pretreated with M-PhSeZnCl (36.2 μM), ROS generation was assessed with the DCFH-DA probe before and after exposure to a bolus of H_2O_2 (264 μM final concentration in the cell medium). Cell uptake of M-PhSeZnCl was verified using a FITC-labeled preparation of the formulation described above.

Cellular GSH

Cellular GSH was assessed by HPLC analysis with fluorescence detection after derivatization with monobromobimane (mBrB, Calbiochem). For GSSG analysis, aliquots of samples were reacted with *N*-ethylmaleimide to mask reduced thiols and then with

dithiothreitol to reduce disulfide bridges, according to [31]. In some experiments colorimetric analysis of total thiol was carried out with the Ellman's assay; briefly, 50 µg of test sample proteins in triplicate were mixed with the Ellman reagent (5,5'-dithiobis(2-nitrobenzoic acid); Sigma-Aldrich; D8130) suspended in PBS, pH 7.5, to a final concentration of 200 µM. After incubation for 5 min at 37 °C, the absorbance was measured at 412 nm and the final concentration of thiols was calculated against a calibration curve of GSH.

GST activity and mRNA expression

GST activity was assessed according to Habig et al. [32] with some minor changes as in [33,34]. GSTP mRNA expression was measured by quantitative RT-PCR analysis as described in [35]. Primers for GSTP were 5'-TCCCAGTTCGAGGGCGGTGT-3' and 3'-CATCTGGGCGGCCTCCCTCT-5'.

Statistics

Data are presented as the mean±SD and differences between series of treatments were assessed using Student's *t* test for paired data and analysis of variance. A probability of *p* < 0.05 was accepted.

Results

Glutathione peroxidase activity of PhSeZnCl

Using the GR-coupled assay procedure of Wendel [24] and keeping the cosubstrates H₂O₂ and GSH steady at final concentrations of 0.21 and 1.0 mM, respectively, initial rates (*V*₀) of PhSeZnCl and ebselen tested at the final concentration of 1 µM were 0.028±0.007 and 0.018±0.005 of absorbance units (Abs)/min (Supplementary Fig. A, *p* < 0.05), which correspond to 0.50 and 0.33 U/ml GPx specific activity, respectively (Supplementary Fig. A, bar chart inset). With this assay procedure, the evaluation of bovine erythrocyte GPx enzyme (0.15 U/ml 0.0187 µM) resulted in a specific activity of 0.20 U/ml.

An almost linear concentration-dependent effect of catalyst concentrations on *V*₀ was observed between 0.25 and 10 µM (Fig. 2A) and the comparison between the test compounds confirmed the higher apparent activity of PhSeZnCl (19,000 Abs/min/M) vs ebselen (7000 Abs/min/M); (PhSe)₂ showed the highest *V*₀ value (31,000 Abs/min/M). This effect of (PhSe)₂ can be explained by the higher contribution of Se equivalents by this compound in the reaction medium, because the catalytic efficiency of this putative product of PhSeZnCl oxidation did not differ significantly from that of ebselen or was even lower after correction for the moles of Se present in the test tube. Assessing the apparent activity of erythrocyte GPx enzyme protein, a concentration-dependent increase in *V*₀ similar to that of ebselen was observed, but at a 100-fold lower molar ratio (Fig. 2A).

The substitution of Se with S in the PhSeZnCl molecule to produce the PhSZnCl analog completely abolished the GPx activity of the compound (data not shown).

Further investigation of apparent activity and verification of reaction specificity were carried out by means of the AR assay. The test performed in the presence of GSH as cosubstrate

provides a direct analysis of H₂O₂ reduction in the GPx mimetic catalysis (Fig. 2B), which confirmed the specificity of reaction for the test molecules and the superiority of PhSeZnCl as a GPx-mimetic compound compared with ebselen.

Reaction kinetics

Reaction kinetics of free PhSeZnCl and M-PhSeZnCl were investigated keeping the concentration of one of the two substrates constant and varying the other, which approximates the analysis to one-substrate Michaelis–Menten reaction kinetics. These Se-organic molecules were compared with bovine erythrocyte GPx, ebselen, the putative oxidation product (PhSe)₂, and the sulfur-containing analog PhSZnCl (Table 1).

Varying the concentration of H₂O₂ (Table 1A), PhSeZnCl showed a K_M for H₂O₂ higher than that of ebselen and similar to that of bovine erythrocyte GPx. (PhSe)₂ showed the lowest affinity for H₂O₂, which was higher only in comparison with M-PhSeZnCl. PhSeZnCl and (PhSe)₂ showed similar V_{max} values that were higher than the corresponding value observed for ebselen.

Assessing the affinity for the substrate GSH (Table 1B), PhSeZnCl showed the lowest K_M value in comparison with ebselen and (PhSe)₂. This was combined with the highest V_{max} (about threefold that of the other compounds). Once microencapsulated, PhSeZnCl showed K_M values for GSH and V_{max} close to those of bovine erythrocyte GPx.

Therefore, reaction kinetics data show that PhSeZnCl has higher V_{max} and catalytic efficiency ($\eta = k_{cat}/K_M$) than ebselen and (PhSe)₂, as calculated varying either the substrate GSH or H₂O₂; importantly, microencapsulation markedly lowered the affinity for GSH and H₂O₂ substrates, reducing both the turnover number and the catalytic efficiency of PhSeZnCl.

Effect of PhSeZnCl on cell viability, apoptosis, and MAPK signaling

Cytotoxicity of PhSeZnCl was investigated by assessing the dose–response effect on the cell viability of cancer cell lines, K562, LnCap, and A549, as well as of GSTP^{+/+} and GSTP^{-/-} allotypes of nontumoral MEFs. MTT and cellular ATP tests were compared as cell viability assays in some cell lines, and corresponding results were obtained (not shown). IC₅₀ values calculated with MTT test are shown in Table 2 and viability curves are shown in detail in Fig. 3 and Supplementary Figs. C and E. PhSeZnCl had the highest inhibitory effect on the GSTP-overexpressing cell line K562 (IC₅₀ of 18.02±1.72 μM), whereas LnCap and A549 cells showed much lower inhibition (IC₅₀>100 μM at 24 h). The low toxicity of this compound in LnCap and A549 was further confirmed after 48 h of incubation with IC₅₀ values of 76.30±7.55 and 41.55±4.06 μM, respectively.

PhSeZnCl showed lower IC₅₀ than ebselen in both the GSTP-overexpressing cell line K562 and MEFs from either the GSTP^{+/+} or the GSTP^{-/-} mice. However, GSTP^{-/-} cells were generally more sensitive to the different Se-organic compounds and, in this context, (PhSe)₂ was the most cytotoxic compound, with ebselen as the less toxic form.

Proapoptotic activity of PhSeZnCl is shown in Supplementary Figs. D1–D3. Short-term treatments (6 h; Supplementary Figs. D1 and D3, left and right top) carried out with 10 μ M PhSeZnCl resulted in higher levels of advanced and late apoptosis in K562 cells compared to MEFs. This effect in K562 cells was higher in the case of PhSeZnCl treatments than in the case of ebselen. Again the proapoptotic effect of PhSeZnCl on MEFs was significantly higher in the GSTP^{-/-} genotype (Supplementary Fig. D3, right). In these cells, ebselen was found to act as a potent proapoptotic agent leading to twofold higher levels of late apoptosis than PhSeZnCl.

In GSTP^{-/-}, but not in the GSTP^{+/+} MEFs, PhSeZnCl stimulated the activity of the MAPK isoforms JNK and ERK (Fig. 4). Baseline activation of these kinases was also observed in GSTP^{-/-} cells.

As assessed through both MTT and clonogenic assays (Supplementary Figs. H and I, respectively) in non tumoral and tumoral cells, microencapsulation mitigated the toxicity of PhSeZnCl. The results of these tests are discussed below under *Cellular effects of M-PhSeZnCl*.

ROS-generating effect of PhSeZnCl

The levels of cellular ROS investigated by the fluorescent probe DCF in various cell models treated with PhSeZnCl showed an inverse relationship with the time- and concentration-dependent decrease in cell viability (Figs. 3 and 5), thus confirming the role of oxidative stress as an underlying event in PhSeZnCl cytotoxicity. In MCF-7 cells (Fig. 5), as well as in K562 and MEFs (not shown), the co-incubation of PhSeZnCl with the antioxidant PEG–CAT revealed that the increase in DCF fluorescence stimulated by the Se-compound almost completely results from an increased generation of H₂O₂, whereas the fluorescence observed in the control tests (untreated cells) does not appear to depend on this species (Fig. 5). The thiol-replenishing agent NAC (Supplementary Fig. F) was particularly efficient at decreasing the fluorescence of DCF in PhSeZnCl-treated as well as in control (untreated) MCF-7 cells (Fig. 5B). The efficacy of NAC as modulator of cellular redox was also demonstrated by the increased capability of MCF-7 cells in reducing the MTT probe used as a cell viability indicator (Fig. 5A). This effect was not observed in the case of PEG–CAT, which did not produce any direct antioxidant protection on this redox-sensitive probe; at the same time, the effects of NAC and PEG–CAT treatments on both the DCF and the MTT probes in MCF-7 cells treated with PhSeZnCl were not cumulative.

In addition to decreasing ROS generation, both antioxidants produced a significant protective effect on the clonogenic expansion of MCF-7 cells that was particularly significant at 10 μ M PhSeZnCl (Supplementary Fig. L).

A lowered generation of cellular ROS was observed in MEFs treated with M-PhSeZnCl in comparison with PhSeZnCl (Supplementary Fig. H, (B) and Fig. 3C). These results are discussed in more detail below under *Cellular effects of M-PhSeZnCl*.

Effects of PhSeZnCl on thiol status and GSTP activity and expression

The effects of PhSeZnCl on cellular thiols and the consequent impact on cytotoxicity were investigated in detail in K562 erythroleukemia cells, which showed high levels of total glutathione (Fig. 6) and GST activity (Fig. 7A and Table 2). The latter was more than 100-fold higher than in MEFs, 0.67 and 87.3 U/mg, respectively, and severalfold higher than in the other cancer cell lines investigated in this study. In fact in a well-recognized cellular model of GST overexpression [36,37], the Pi form largely predominates over other isoenzymes [38]. In these cells, PhSeZnCl was confirmed to deplete both the reduced and the oxidized forms of glutathione (Fig. 6) and was found to have much higher GST inhibition (Fig. 7B and Supplementary Fig. E) and proapoptotic (Supplementary Fig. D) activity than ebselen. These findings are in agreement with the effects observed for these Se-organic molecules on cell viability discussed above and shown in Table 2. In fact, the inhibition of GST activity observed in K562 cells at increasing concentrations of the two test compounds correlated linearly with the observed decrease in cell viability (Fig. 7B). The data of Fig. 7B and Supplementary Fig. E clearly show that the extent of PhSeZnCl cytotoxicity in this erythroleukemia cell line starts to differentiate from that of ebselen when compound concentrations produce a significant inhibition of GST activity, thus showing the *in vitro* pharmacological threshold (between 10 and 50 μ M) for the drug resistance role of this protein.

GST activity inhibition by PhSeZnCl treatment was also observed in non-cancerous GSTP^{+/+} MEFs in the absence of significant changes of either protein or mRNA levels of GSTP (Fig. 7C).

GSH and GSSG depletion after PhSeZnCl treatment was also observed in MEFs and was more pronounced in the GSTP^{-/-} genotype, which showed lowered baseline levels of total glutathione if compared with GSTP^{+/+} cells (Fig. 6) and a GSH/GSSG ratio close to that found in K562 cells (Fig. 6). Treatment of K562 cells or MEFs with NAC produced a marked increase on cellular thiols (from 2- to 10-fold) assessed by the Ellman reaction test, either in the baseline control test or after PhSeZnCl treatments (Supplementary Fig. F). The thiol-replenishing effect of NAC was observed to be fast and particularly efficient if operated posttreatment with PhSeZnCl (not shown). The protection or even implementation of cellular thiols during PhSeZnCl treatment resulted in a lower production of ROS (as DCF fluorescence) and consequently in a lower decrease in cell viability, which are described under *ROS-generating effect of PhSeZnCl* and in Fig. 5. PEG-CAT did not produce a significant effect on thiol levels either before or after PhSeZnCl treatment in MEFs and K562 cells.

Cellular effects of M-PhSeZnCl

Toxicity and cell uptake of M-PhSeZnCl were studied in MCF-7 cells and MEFs. As far as cell uptake, an increased fluorescence was observed in FITC-labeled M-PhSeZnCl treated MCF-7 cells after 4 h of incubation at 37 °C (Supplementary Fig. F, trace in red in (B)), thus confirming the cellular delivery of the formulation. The time course of cell uptake (Supplementary Fig. G, (C)) showed a plateau of fluorescence reached after 15 h of treatment.

In GSTP^{+/+} and GSTP^{-/-} MEFs treated for 5 h with FITC-labeled M-PhSeZnCl (15 μ M final concentration of the test compound; Supplementary Fig. G, (D)), the formulation was taken up without qualitative and quantitative differences in the two allotypes, and M-PhSeZnCl fluorescence was found to localize in the cytoplasm under the form of organized bodies. This finding suggests that the uptake of M-PhSeZnCl may occur by an endocytosis-like process. In addition to morphology data, the uptake of M-PhSeZnCl (up to PhSeZnCl compound concentrations of 100 μ M) did not produce any appreciable effect on cell viability or ROS levels in GSTP^{+/+} MEFs, whereas a slight decrease of cell viability was observed in association with a significant decrease of ROS production in GSTP^{-/-} MEFs (Supplementary Fig. H, (A and B)).

Cell viability data of MCF-7 cells assessed at 24 h (Supplementary Fig. H, (C)) confirmed the significantly lower toxicity of the microencapsulated formulation in comparison with the free form of PhSeZnCl and this difference between the two forms of the test compound was maintained at 48 h of treatment (Supplementary Fig. H, (D)) when the decrease in viability for M-PhSeZnCl reached a maximum of approximately 40% at the final concentration of the test compound of 100 μ M.

The cellular protection effect of PhSeZnCl microencapsulation was also investigated in human nontumoral BEAS and tumoral MCF-7 cells by clonogenic assay (Supplementary Fig. I, (A and B), respectively). To highlight the effect of the microencapsulation on PhSeZnCl cytotoxicity, the assay was performed at either low (moderately toxic) or high (very toxic) in vitro concentrations of the test compound, namely 10 and 100 μ M. The results clearly confirmed that microencapsulation protects nontumoral BEAS cells from the toxicity of PhSeZnCl, and this effect was significantly higher than the effect produced by the lowering of PhSeZnCl concentrations from 100 to 10 μ M (Supplementary Fig. I, (A)). This finding was also confirmed in MCF-7 cells (Supplementary Fig. I, (B)) that, in addition were treated with M-PhSeZnCl after being transfected with different allelic variants of the GSTP1-1 enzyme to assess the GSTP1-1-dependent antioxidant and cell protection properties of PhSeZnCl upon exposure to exogenous H₂O₂ (Fig. 8).

In transfected MCF-7 cells, at all the experimental time points of the treatment with M-PhSeZnCl (36.2 μ M), the levels of ROS were lowered in the subsets transfected with A or B variants (data not shown). This effect lasted until 17 h of treatment in the case of cells transfected with the variant A. The cells transfected with GSTP1-1 allelic variants, but not with the vector or the inactive form of the enzyme Y7F, responded to M-PhSeZnCl pretreatment with a lowered burst of ROS after exposure to H₂O₂ (264 μ M) (Fig. 8). This effect was higher in cells transfected with the most active variants (efficacy increased in the order B<D<A<C).

Discussion

The anticipated potential of PhSeZnCl as a GPx-mimetic agent [8] has been confirmed in this study. Apparent initial rates calculated with different assay procedures were the highest compared with the prototypical Se-organic drug ebselen [10] and (PhSe)₂, an oxidation product of PhSeZnCl used as a lead compound for the production of novel GPx-mimetic

molecules [5]. Direct evidence of the H₂O₂-reducing activity of PhSeZnCl was obtained with an AR-HRP coupled test, and the substitution of Se with S completely abolished the GPx activity of this Se-organic molecule, thus confirming the superiority of Se over S as a hydroperoxide reduction catalyst proposed in either small-molecule GPx-mimetic compounds [39] or GPx enzyme proteins in which the catalytic SecGPx can reduce hydroperoxides with rate constants of at least 1 order of magnitude higher than CysGPx's (10^7 vs 10^6 M⁻¹ s⁻¹, respectively) [9].

Kinetic parameters of PhSeZnCl (Table 1), however, demonstrated the same catalytic efficiency ($\eta = k_{\text{cat}}/K_M$) for both cosubstrates combined with a higher turnover number (k_{cat}) and the lowest K_M for GSH in the series of test molecules. Such kinetics data, however, were far from those of bovine erythrocyte GPx. Altogether these results suggest a propensity for PhSeZnCl to behave as a rather non-specific reactant of cellular thiols, which may limit the chances of developing its pharmacological applications as a GPx mimetic and cellular antioxidant.

In fact, the characteristic reactivity of this and other forms of organic Se with cellular thiols and the stimulation of H₂O₂-generating pathways confirmed in this study represent major concerns, being the cause of severe “stressogenic” events reviewed in [15–17,40]. On the other hand, this chemistry can be a useful prerequisite in designing efficient redox catalysts for different chemical and biological destinies [7], as well as anticancer drugs with therapeutic applications in chemoresistant cancers, particularly those featuring an enhanced expression of GSH-related genes. Here, the superfamily of GSTs plays a major role, being one of the main players in drug-resistance mechanisms [41].

In this respect, inhibition of GST enzyme activity and signaling function could represent a key event in the thiol-reactive activity of Se-organic molecules [21] and, according to earliest evidence obtained in K562 erythroleukemia cells [42], GST targeting may confer selective anticancer properties to these molecules and particularly to the thiol-reacting properties of PhSeZnCl. In fact, in this cell model of GST-hyperexpressing malignancy [36,37] in which the Pi class represents 2/3 of the total enzyme activity [38], with the Mu class secondary and the anionic Alpha class present only in trace amounts ($\approx 3\%$), PhSeZnCl was a more efficient inhibitor of GST activity than ebselen. Cell-free experiments with crude preparations of placental and red blood cell GSTP enzyme protein confirmed that Se-organic drugs are irreversible inhibitors of GSTP and that the inhibitory activity of PhSeZnCl is superior to that of ebselen and even (PhSe)₂ (D. Bartolini et al., unpublished data).

The relationship between the inhibition of GST activity and the cell viability in K562 cells was linear in the case of ebselen and exponential in the case of PhSeZnCl (Fig. 7B and Supplementary Fig. E), with an IC₅₀ value of 18.0 ± 71.7 μM calculated by cell viability data.

Cell viability inhibition of PhSeZnCl was significantly lowered (IC₅₀ > 50 μM) in the epithelial carcinomas LnCap, MCF-7, and A549, which showed approximately 10- to 20-fold lower levels of GST than K562 cells, as well as in non-tumoral cells such as BEAS and MEFs that express 100-fold lower GST activity in the cytosol (Table 2).

Perhaps as a consequence of GST inhibition, PhSeZnCl produced in K562 cells and MEFs a severe depletion of both the reduced and the oxidized form of glutathione (Fig. 6), which further demonstrates the centrality of thiol metabolism in PhSeZnCl cytotoxicity. Along with Se-S adduct formation, direct oxidation effects by the increased flux of ROS and events of leakage or active export of GSH or GSSG promoted by the execution of the apoptotic program [43] are all possible underlying events of this severe dyshomeostasis of cellular thiols. Indeed, the thiol-restoring agent NAC and the H₂O₂ scavenger PEG-CAT prevented at least in part the oxidative stress-associated cytotoxicity of PhSeZnCl in various human cell models such as MCF-7 and BEAS, as well as in MEFs.

The central role of GST in preventing thiol depletion and cytotoxicity of PhSeZnCl was further demonstrated in GSTP1-knockout MEFs, which were also characterized by the presence of lowered baseline levels of total glutathione. In these cells, the proposed signaling function of GSTP1 reviewed in [25,44–46] was demonstrated to play a role in PhSeZnCl cytotoxicity, influencing the activation of JNK and ERK, two key MAPK isoforms at the crossroads between cellular stress and proliferation/death signaling, see [27,28] and references therein.

Altogether these findings reveal that GSTP protein can represent on one hand a major sacrificial target and protection system against the toxicity of Se-organic molecules, and on the other hand it can act as a sensor and redox signaling node for these compounds. The modulation of GSTP signaling by Se-derived molecules may have implications to drug-resistance mechanisms [47] as well as in the development of GSTP-targeted therapeutic protocols to treat bone marrow hypoproliferative conditions, such as myelodysplastic syndromes [45] and uremic anemia [34], a condition associated with GSTP overexpression, lowered hematopoietic potential, and oxidative stress [48].

The targeting of GSTP redox signaling with PhSeZnCl and other Se compounds needs to operate under non-toxic conditions. To this end, a microparticle formulation of PhSeZnCl with lowered GPx-mimetic and thiol-reacting potential was developed and tested for protection effects in a cellular model of oxidative stress. The decreased cytotoxicity of this formulation was demonstrated by cell viability and clonogenic tests in human cancerous and noncancerous cells and can be straightforwardly explained by the lowered reactivity with cellular thiols. Moreover, M-PhSeZnCl uptake studies in MCF-7 cells and MEFs showed cellular dynamics that may further contribute to controlling PhSeZnCl cytotoxicity. A cytosolic localization of a FITC-labeled formulation of M-PhSeZnCl in organized bodies that resemble those formed in macrophages exposed to microparticle material [49] was observed, thus suggesting an endocytosis-like process that will be further investigated as regards its possible affects on PhSeZnCl activity/toxicity.

This absence of toxicity provided the prerequisite to demonstrate that M-PhSeZnCl prevents the burst of DCF fluorescence induced by H₂O₂ exposure in MCF-7 cells transfected with different allelic variants of GSTP1-1. According to recent findings by some of us [22], this effect points to a mechanism of cellular protection against an event of oxidative stress, which is enhanced in the presence of most active GSTP1-1 variants in the cytosol. Mechanistic explanations for such a GSTP-dependent protection effect of M-PhSeZnCl may

include redox regulation of ROS-scavenging proteins such as Prdx6 [22]. This one-Cys Prdx, associated with the cell membrane, shows peroxidase activity that is regulated through heterodimerization with GSH-loaded GSTP1. The GSH activated by binding to GSTP (GS⁻) serves as a source of electrons for reduction of the Prdx6 catalytic Cys47 [50,51].

Conclusion

The GPx mimetic PhSeZnCl is a very effective reagent of cellular thiols and a cytotoxic agent with possible applications in the chemotherapy of drug-resistant cancers. Along with glutathione, Cys residues of the detoxifying enzyme and signaling protein GSTP are an elective target of this compound. As a consequence, the expression pattern of GSTP in tumoral and non-tumoral cells influences the anticancer cytotoxic and redox signaling effects of PhSeZnCl.

The data in this study demonstrate that microencapsulation is a relatively simple strategy to control such thiol reactivity and the oxidative stress effects of PhSeZnCl. This is a straightforward solution to allow applications alternative to anticancer therapy, which may include the modulation of GSTP-dependent signaling pathways associated with the stress response, cell cycle regulation, and antioxidant protection. This original observation may lead to overcoming the GPx-mimetic paradigm proposed so far for Se-organic drugs to open up a path to the more pragmatic concept of GSTP-signaling modulators.

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Fig. 1.

Structure and possible catalytic mechanism of PhSeZnCl. (A) Structure and incipient oxidation state of the Se moiety of phenyl-Se-Zn-Cl (PhSeZnCl) shown in comparison with the catalytic pocket of glutathione peroxidase (GPx) enzyme. Here the Se atom is coordinated with the triad Gln-Trp-Sec (right inset). In both cases, Se is maintained in the oxidation state -1 . (B) Proposed catalytic mechanism for the GPx-mimetic activity of PhSeZnCl. Peroxide reduction reaction proceeds through the formation of an unstable selenenic acid intermediate (PhSeOH), which reacts with GSH to generate the selenenyl sulfide intermediate PhSeSG. A second GSH molecule reacts with the Se-S bond regenerating the active selenol site and thus producing a molecule of oxidized GSH (GSSG).

Fig. 2.

Apparent initial rates of the GPx mimetic compounds PhSeZnCl, ebselen, and (PhSe)₂. (A) Apparent initial rates of the Se-organic compounds were assessed by the GR-coupled assay [24] varying the final concentrations of the catalysts between 0.25 and 10 μM. Bovine erythrocyte GPx enzyme was used as a reporter test (diamond symbol and dashed regression line). (B) H₂O₂ reduction activity of the Se-organic catalysts PhSeZnCl and ebselen (10 μM) was assessed using the fluorescent probe Amplex red. *p<0.05 vs PhSeZnCl treatment.

Fig. 3.

Effects of PhSeZnCl on cell viability and ROS production in (A) K562 human erythroleukemia cells and nontumor MEFs with (B) GSTP^{+/+} or (C) GSTP^{-/-} isotypes. Concentration and time-dependent effects of PhSeZnCl on cell viability were assessed by MTT test and, in the same experiments, cytosolic levels of ROS (iROS) were measured by the fluorescent probe DCF (insets). *p<0.05.

Fig. 4.

Protein expression and activity of JNK and ERK1/2 in GSTP^{+/+} and GSTP^{-/-} MEFs treated with PhSeZnCl. The cells were exposed to 100 μ M PhSeZnCl for 6 h and phosphorylated and nonphosphorylated forms of these MAPKs were assessed by immunoblotting. *p<0.05 or higher vs control experiments.

Fig. 5. Effects of NAC and PEG-CAT on (A) cell viability and (B) normalized DCF fluorescence in MCF-7 cells treated with PhSeZnCl. Antioxidants were coadministered with the Se-organic compound during the entire time of treatment (24 h). *** $p < 0.0005$ (t test).

Fig. 6.

Levels of reduced glutathione and glutathione disulfide in K562 erythro-leukemia cells and GSTP^{+/+} and GSTP^{-/-} MEFs treated with PhSeZnCl. Cells were treated with 100 μ M PhSeZnCl for 24 h and thiols measured in cell homogenates by HPLC analysis coupled with fluorescence detection after mBrB derivatization. * $p < 0.05$ or higher vs control experiments. $p < 0.05$ vs GSTP^{-/-} MEFs; # $p < 0.01$ vs GSTP^{+/+} MEFs. iMEFs, immortalized MEFs.

Fig. 7.

Effects of PhSeZnCl on GST activity in K562 erythroleukemia cells and nontumor GSTP^{+/+} and GSTP^{-/-} MEFs. (A) Baseline GST activity was assessed in cell lysates of K562 and GSTP^{+/+} MEFs. (B) Then the 24-h dose-dependent effects of PhSeZnCl and ebselen on GST activity and cell viability were compared by regression analyses in K562 cells. In this fitting, the concentrations of Se-organic compounds decreased from right-top to left-bottom and were as in Supplementary Fig. E. (C) 24-h treatment effects on GST activity with 100 μ M PhSeZnCl were assessed in GSTP^{+/+} MEFs (left top) together with GSTP mRNA and protein expression levels (left bottom and right, respectively).

Fig. 8.

Effects of M-PhSeZnCl on DCF fluorescence of MCF-7 cells transfected with different allelic variants of GSTP1-1 and challenged with H_2O_2 . MCF-7 cells transfected with allelic variants of GSTP1-1 (from A to C) as reported in [22] were pretreated with M-PhSeZnCl (36.2 μ M) for 17 h and then were exposed to H_2O_2 (264 μ M). The production of cellular ROS was assessed through the real-time kinetics measurement of DCF fluorescence. Cells transfected with empty vector or the catalytically inactive Y7F were used as controls.

Table 1

Catalytic parameters of GPx-like reaction kinetics. (A) Varying $[H_2O_2]$ between 0 and 0.88 mM, keeping $[GSH]$ steady at 1 mM.

Test molecule	$H_2O_2 V_{max} (M s^{-1}) \times 10^{-7}$	$H_2O_2 K_M (M) \times 10^{-4}$	$K_{cat} (s^{-1})$	$\eta (M^{-1} s^{-1}) \times 10^3$
GPx	38 ± 12	2.4 ± 0.7	205.0	854.2
Ebselen	35 ± 2	1.1 ± 0.4	0.4	3.6
PhSeZnCl	162 ± 3	1.9 ± 0.6	1.6	8.4
(PhSe) ₂	158 ± 7	8.0 ± 0.4	1.6	2.0
M-PhSeZnCl	28 ± 8	13.0 ± 4.0	0.3	0.2

(B) Varying $[GSH]$ between 0 and 4 mM, keeping steady $[H_2O_2]$ at 0.21 mM

Test molecule	$GSH V_{max} (M s^{-1}) \times 10^{-7}$	$GSH K_m (M) \times 10^{-4}$	$K_{cat} (s^{-1})$	$\eta (M^{-1} s^{-1}) \times 10^3$
GPx	20 ± 0	13.0 ± 0.1	107.0	82.0
Ebselen	20 ± 8	1.4 ± 1.0	0.2	1.4
PhSeZnCl	58 ± 5	0.6 ± 0.1	0.6	10.0
(PhSe) ₂	20 ± 2	1.5 ± 0.9	0.2	1.3
M-PhSeZnCl	25 ± 8	18.6 ± 15.8	0.3	0.2

The reduction of H_2O_2 by GSH in the GR-coupled method was investigated. The Se-organic drugs were tested at the final concentration of 10 μM and bovine erythrocyte GPx concentration was 0.0187 μM . K_{cat} and η values were calculated from mean values of other kinetics parameters.

Table 2

Cell viability inhibition (IC₅₀ μ M, 24 h) by the Se-organic drugs and baseline GST enzyme activity in some cancerous and noncancerous cell lines.

	<u>Se-organic molecules</u>			GST activity (U/mg protein)
	PhSeZnCl	Ebselen	(PhSe) ₂	
Cancerous cells				
LnCap	> 100	–	–	–
A549	> 100	> 100	> 100	11.9 ± 1.3
MCF-7	81.2 ± 4.0	–	–	3.9 ± 0.8
K562	18.0 ± 1.3	51.0 ± 3.3	92.4 ± 3.2	86.9 ± 0.7
Noncancerous cells				
MEF GSTP ^{+/+}	84.9 ± 3.0	> 100	83.4 ± 5.7	0.67 ± 0.10
MEF GSTP ^{-/-}	69.9 ± 4.0 [*]	87.3 ± 2.5	47.4 ± 5.6 [*]	0.01 ± 0.00
BEAS	53.7 ± 0.4	78.7 ± 0.5	65.9 ± 0.6	–

* $p < 0.05$ or higher vs ebselen and vs GSTP^{+/+}.