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Comparison of different selenocompounds with respect to nutritional value *vs.* toxicity using liver cells in culture☆

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Abstract

The essential micronutrient selenium (Se) exerts its biological effects mainly through enzymatically active selenoproteins. Their biosynthesis depends on the 21st proteinogenic amino acid selenocysteine and thus on dietary Se supply. Hepatically derived selenoprotein P (SEPP) is the central selenoprotein in blood controlling Se transport and distribution. Kidney-derived extracellular glutathione peroxidase is another relevant serum selenoprotein depending on SEPP for biosynthesis. Therefore, secretion of SEPP by hepatocytes is crucial to convert nutritional sources into serum Se, supporting Se status and selenoprotein biosynthesis in other tissues.

In order to compare the bioactivity of 10 different selenocompounds, their dose-dependent toxicities and nutritional qualities to support SEPP and glutathione peroxidase biosynthesis were determined in a murine and two human liver cell lines. Characteristic dose- and time-dependent effects on viability and SEPP production were observed. Incubations with 100 nM sodium selenite, L- or DL-selenocystine, selenodiglutathione or selenomethyl-selenocysteine increased SEPP concentrations in the culture medium up to 6.5-fold over control after 72 h. In comparison, sodium selenate, L- or DL-selenomethionine or methylseleninic acid was less effective and increased SEPP by 2.5-fold under these conditions. As expected, ebselen did not increase selenoprotein production, supporting its classification as a stable selenocompound. Methylseleninic acid, L-selenocystine, selenodiglutathione or selenite induced cell death in micromolar concentrations, whereas selenomethionine or ebselen was not toxic within the concentration range tested.

Our results indicate that hepatic selenoprotein production and toxicity of selenocompounds do not correlate with and rather represent compound-specific properties. The favourable profile of selenomethylselenocysteine warrants its consideration as a promising option for supplementation purposes.

Keywords: Micronutrient; Trace element; Selenoprotein P; Selenium; Toxicity; HepG2

1. Introduction

Selenium (Se) is an essential micronutrient which exerts its biological effects mainly *via* selenocysteine (Sec)-containing proteins [1]. The Sec residue typically is located in the catalytic site of enzymatically active selenoproteins, thus representing an essential component of, e.g., glutathione peroxidases (GPX), thioredoxin reductases or iodothyronine deiodinases [2,3]. Transport and storage of Se are mainly exerted by the liver-derived circulating glycoprotein selenoprotein P (SEPP) which contains up to 10 Sec residues per molecule and accounts for the majority of Se in human and rodent blood [4]. Se displays a U-shaped curve of efficiency, and both pathologies caused by Se poisoning and deficiencies from insufficiently low daily intake, absorption defects or inadequate parenteral nutrition have been described [5–10].

In general, Se concentrations within the different tissues are hierarchically controlled and the pathways involved safeguard a sufficient supply of the micronutrient to the most essential, vulnerable and important organs including the brain, testes and endocrine glands [11–14]. In addition, the different selenoprotein transcripts depend on Se to a different extent during translation, and very essential selenoproteins are prioritized in times of Se shortage giving rise to a second important hierarchy of Se supply in mammals [14–17]. A specific translation initiation factor, i.e., eIF4a3, has recently been identified to participate in the transcript-specific Se channelling [18]. The tissue-specific Se status and preferential supply to, e.g., the brain have been shown to critically depend on SEPP expression and circulating SEPP concentrations [19–22]. Several studies have also indicated that biosynthesis of the mainly kidney-

Abbreviations: GPX, glutathione peroxidase; GSSeSG, selenodiglutathione; MeSeA, methylseleninic acid; Se, selenium; Sec, selenocysteine; $SeCys_2$, selenocystine; SeMet, selenomethionine; SeMeSeCys, selenomethylselenocysteine; SEPP, selenoprotein P (human); SepP, selenoprotein P (mouse).

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derived extracellular GPX isoform, i.e., GPX3, depends on circulating SEPP for regular expression [21,23,24].

Studies in humans and animals have described unequal efficiencies of the different selenocompounds with regard to raising tissue and blood Se concentrations. In general, selenomethionine (SeMet)-based supplements are apparently more effective compared to selenite- or selenate-based ones [25–30]. One reason for this striking difference is given in the random insertion of SeMet instead of methionine (Met) into all proteins in response to AUG codons. This effect is caused by the ribosome failing to distinguish between SeMet- and Met-loaded tRNA during translation [31]. Hereby, total Se concentrations in plasma can be raised without limits and independent of the biosynthesis of real selenoproteins to potentially toxic levels [32]. Another reason may be found in the metabolic profile and capacity of hepatocytes which control Se status and blood Se concentrations *via* SEPP production and secretion [21,33,34]. The different selenocompounds might differ in their ability to be absorbed by the gastrointestinal tract and taken up and metabolized by hepatocytes in order to be converted into SEPP and used to increase serum Se concentrations and tissue Se supply. Therefore, we have chosen a number of different selenocompounds known from nutrition, supplements or pharmacological use, and tested their ability to support and increase SEPP production by human and murine hepatocytes in culture. Our data highlight that there are pronounced differences between the different selenocompounds with respect to their toxicity and anabolic value in raising hepatic selenoprotein biosynthesis.

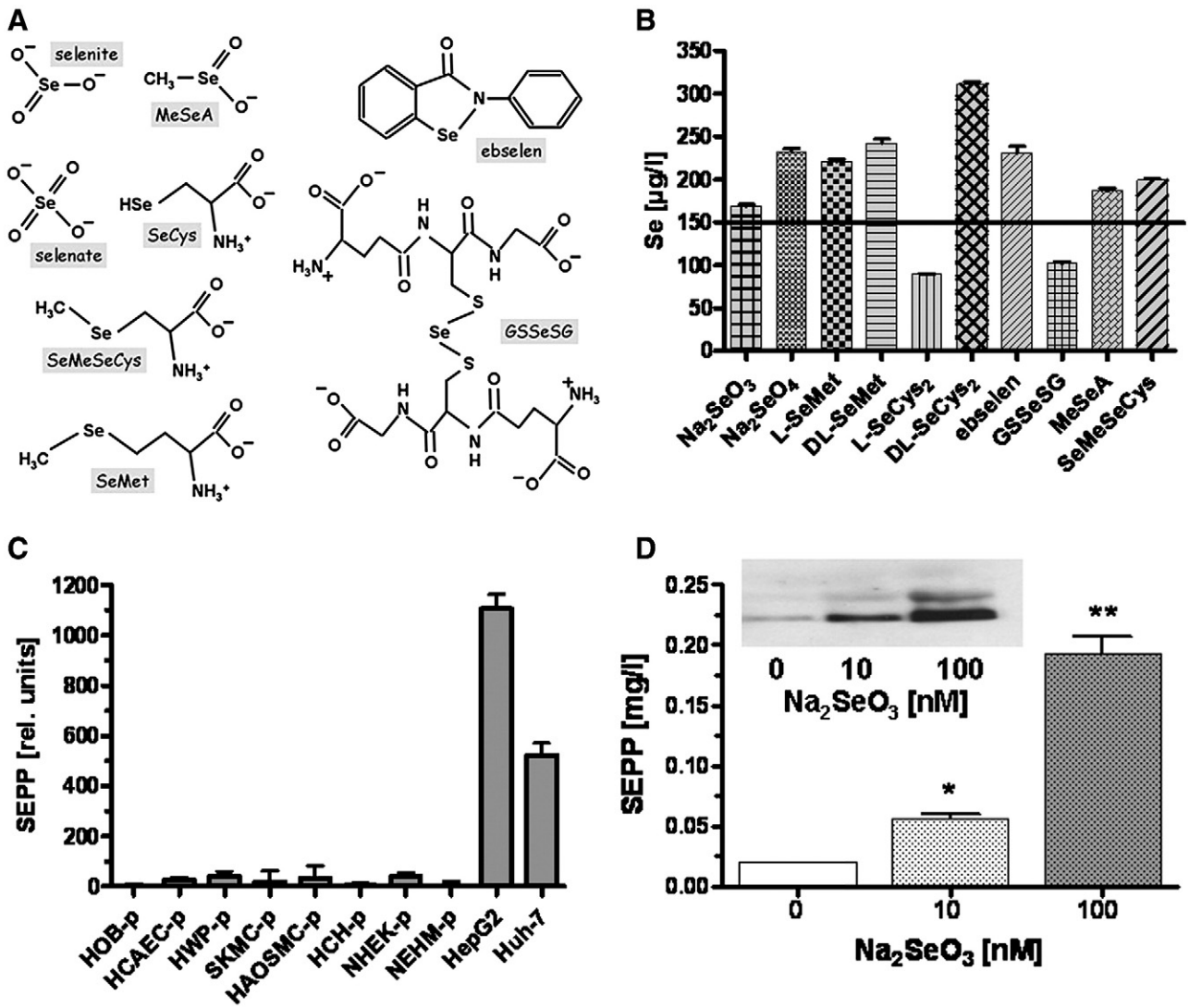

Fig. 1. Overview of selenocompounds, cell culture and SEPP quantification. (A) Structures and abbreviations of selenocompounds used in this study. Na_2SeO_3 and Na_2SeO_4 are naturally occurring inorganic Se salts. GSSeSG is generated during the reductive metabolism of Na_2SeO_3 and Na_2SeO_4. SeCys represents the 21st proteinogenic amino acid and $SeCys_2$ the corresponding disulfide. SeMet is synthesized by plants and often used as a supplement in human studies. SeMeSeCys and MeSeA are described as potent anticarcinogenic selenocompounds. Ebselen is used in clinical applications as a glutathione peroxidase mimetic. (B) Characterization of selenocompound stock solutions prior to adjustment. The selenocompounds were dissolved in water or DMSO and diluted to a final nominal Se concentration of 150 µg Se/L (black horizontal bar). Fluorimetric analyses revealed considerable deviations from the target concentrations leading to respective adjustments of the stock solutions and repeated analysis prior to experimental use. Data are mean±S.E.M. of triplicate measurements. (C) Screening of different human cell lines for SEPP expression. Conditioned cell culture media of different primary cells along with the human hepatocellular carcinoma cell lines HepG2 and Huh-7 were analysed for SEPP production. Data represent means±S.E.M. of duplicate measurements. HOB, Normal human osteoblasts; HCAEC, normal human coronary artery endothelial cells; HWP, normal human white preadipocytes; SKMC, normal human skeletal muscle cells; HAOSMC, normal aortic smooth muscle cells; HCH, normal human chondrocytes; NHEK, normal human keratinocytes foreskin; NEHM, normal human epidermal melanocytes. (D) Characterization of the quantification method for secreted SEPP. HepG2 cells were stimulated with 0, 10 or 100 nM Na_2SeO_3 for 72 h and conditioned cell culture media ($n=3$) were analysed. A representative Western blot after cell culture protein precipitation is shown (top) along with results of the luminometric SEPP quantification assay (bottom); ANOVA and Dunnett's T3 test: *$P<.05$, **$P<.01$.

2. Methods and materials

2.1. Materials

All chemicals were of analytical grade and obtained from Sigma-Aldrich (München, Germany) or Merck (Darmstadt, Germany). Cell culture materials were obtained from Sarstedt (Nümbrecht, Germany), Invitrogen (Karlsruhe, Germany) or Biochrom AG (Berlin, Germany). Selenocompounds were purchased from Sigma-Aldrich or PharmaSe (Lubbock, TX, USA). A schematic overview of the compounds tested is provided (Fig. 1A). Stock solutions of the different selenocompounds were prepared as follows: all selenocompounds except for ebselen were dissolved in PBS. Ebselen was dissolved in DMSO and diluted with PBS to a final concentration of less than 1‰ DMSO in cell culture. Sterile filtration of the compounds was achieved using 0.2-μm filter devices (Whatman, Schleicher & Schuell, Germany). Se concentrations in the stock solutions used for the experiments were measured and adjusted according to the actual Se contents of these preparations (Fig. 1B).

2.2. Fluorimetric Se determination

Quantification of Se was performed by a fluorimetric method *via* piazselenol formation with 2,3-diaminonaphtalene (DAN) as described [35,36]. Dilutions of a commercial Na_2SeO_3 standard solution (Sigma-Aldrich) were used to construct a calibration curve. A human serum standard (Seronorm, Sero AS, Billingstad, Norway) was used as reference to validate the method yielding interassay and intra-assay variations of <10% during the measurements.

Selenocompound stock solutions were analysed by digesting 100 μl with 500 μl of a mixture of perchloric and nitrous acid [1:4 (v/v); HNO_3 (65%)/$HClO_4$ (70%)] for 2 h at 190°C to release the contained Se. After HCl treatment, EDTA and DAN were added and the piazselenol generated was extracted at room temperature (RT) with cyclohexane. Fluorescence was determined in glass microcuvettes with a fluorescence spectrophotometer (LS50B, PerkinElmer; λexcitation: 364 nm, λemission: 520 nm) and analysed as described [20].

2.3. Cell culture

Conditioned cell culture media from primary cells tested for SEPP biosynthesis were kindly provided by Promocell (Heidelberg, Germany). The three immortalized cell lines (HepG2, Huh-7, Hepa 1–6) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and the Japanese Collection of Research Bioresources, respectively. Cells were routinely maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum in tissue culture flasks at 37°C and 5% CO_2. Culture medium was exchanged at 3-day intervals and cells were passaged on a weekly basis, using a 1:5 splitting ratio. In order to prepare for Se treatments, cells were counted using a haemocytometer and seeded at a concentration of 5×10^6 cells per T75 cell culture flask and grown to 70% confluence. After 24 h, all medium was removed and cells were washed twice with PBS. Cells were stimulated with the indicated concentrations of various selenocompounds in serum-free medium. The conditioned culture media were collected and analyzed for SEPP concentrations after the indicated periods of incubation time. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed in quadruplicates with 2×10^4 viable HepG2 cells/well in 96-well plates. All selenocompounds were tested in a concentration range of 0.1 nM to 1.0 mM and viability was determined according to the protocol of Denizot et al. [37]. Briefly, cells were incubated in the presence of MTT and analyzed for their ability to generate a purple formazan dye. After incubation for 24, 48 or 72 h, the absorbance was measured using a microplate reader (400 ATX ELISA Reader, SLT-Labinstruments, Achterwehr, Germany) at a wavelength of 595 nm and the ratio of viable cells was calculated.

2.4. Western blot analysis

SEPP expression was analyzed by Western Blot analysis or by an immunoluminometric sandwich assay as described [38]. The concentration of SEPP in the culture medium under these test conditions proved to be too low to yield a detectable Western signal straight from crude cell culture supernatants. Therefore, proteins were precipitated with cold acetone prior to Western blot analysis. The protein pellet was resuspended in 1/10 the original volume, and equal volumes were added to each slot. Proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany), verified with respect to uniform loading and complete transfer by Ponceau S staining of the membrane and incubated with anti-SEPP antiserum as described earlier [38,39]. Dot blot analyses were conducted from the supernatants of murine Hepa 1–6 cells after seeding 2×10^4 cells/200 μl medium into 96-well-plate cavities. After 24 h of adhesion time, cells were stimulated with different concentrations of the selenocompounds in FCS-free media for a time period of 72 h. Then, 100 μl of the conditioned culture media was blotted directly onto nitrocellulose membranes by a dot blot device (Bio-Dot SF Microfiltration Apparatus, BIO-RAD). Primary antisera against murine SepP have been generated and described earlier [21,40]. HPR-conjugated secondary antibodies (Sigma) were used in combination with an ECL-based detection system (Thermo Scientific Pierce, Schwerte, Germany) and X-ray films (Amersham) to visualize the Western blot signals. Quantification was achieved by densitometry of the signals using the ImageJ software (National Institutes of Health).

2.5. SEPP quantification and preanalytic characterization of SEPP in culture medium

The immunoluminometric sandwich assay for quantification of SEPP from human sera has been developed recently and was used directly with cell culture supernatants. Functional assay characteristics and the limit of detection were as described previously [38]. Stability of SEPP in cell culture medium was determined at room temperature and after samples were subjected to multiple freeze (liquid nitrogen) and thaw (room temperature until melted completely) cycles as described with human serum samples [38].

2.6. GPX1 activity measurements

The enzymatic activity of GPX1 was determined from HepG2 cell homogenates after stimulation with the selenocompounds as described earlier [20]. Briefly, cells were seeded, cultivated, washed with PBS and stimulated with 100 nM of the various selenocompounds in serum-free medium in T75 cell culture flask as described above. After 72 h, cells were collected, washed, homogenized and analysed in triplicate by a coupled photometric test using *t*-butylhydroperoxide as substrate [41].

2.7. Statistical analysis

GraphPad Prism 4 software and SPSS 16 were used for all computations with the tests indicated in the figure legends. Data are expressed as mean±S.E.M. Statistical significance was defined as $P<.05$ (*), $P<.01$ (**) or $P<.001$ (***).

3. Results

3.1. Preparation of the selenocompound stock solutions

Selenocompounds (Fig. 1A) were obtained from different sources, dissolved in water or DMSO (in the case of ebselen), and stock solutions were adjusted to identical Se concentrations. Initially, stock solutions were diluted to a final concentration of 150 μg Se/L according to the information on the data sheets and the weights determined during stock preparations. Next, total Se concentrations were determined in order to verify that equivalent concentrations of Se were contained in the different stock solutions. Deviations from the expected concentrations were noted (Fig. 1B). Therefore, stock solutions were re-adjusted accordingly and correct Se concentrations were verified by fluorimetric Se determination.

3.2. Evaluation of different cell lines for their ability to express and secrete SEPP

In order to identify a suitable cell line for our studies, the NCBI database was searched for publications describing SEPP-expressing cell lines. Many positive references were identified describing SEPP mRNA in a number of well-characterized liver, thyroid, brain, breast, bone, prostate, kidney or gastrointestinal cell lines. But permanent cell lines might lose their ability to express and secrete high SEPP protein levels during prolonged cultivation and propagation cycles. Accordingly, our analyses with a number of these cells were negative and yielded none or very little immunoreactive SEPP from these cell lines with the exception of liver cells. Therefore, conditioned cell culture media from a number of human primary cells of different origins were screened for SEPP production (Fig. 1C). Again, even these primary cells had to be classified as SEPP negative in comparison to hepatic HepG2 or Huh-7 cells. SEPP expression of the latter could easily be quantified directly by the immunoluminometric assay. Given that liver is the major site of nutritional Se metabolism and SEPP production, the corroboration of the human hepatic cell lines Huh-7 and HepG2 as the most suitable systems for SEPP analysis appeared physiologically meaningful. These cells were thus selected for the comparative analysis of the different selenocompounds.

3.3. Preanalytical characterization of SEPP in culture media

In order to validate the immunoluminometric assay for quantification of SEPP from cell culture media, HepG2 cells were incubated with increasing amounts of Na_2SeO_3 in FCS-free medium for 72 h. Se-dependent SEPP production was detected by Western blot analysis as a pattern of two bands and by the immunoluminometric SEPP assay (Fig. 1D). Under the conditions chosen, SEPP increased in a dose-dependent manner in the conditioned media by twofold in response to 10 nM Na_2SeO_3 and by eightfold in response to 100 nM Na_2SeO_3 as compared to control. The semi-quantitative Western blot analysis was in good agreement with the data from the quantitative immunoluminometric SEPP assay.

Next, stability of SEPP was determined in conditioned cell culture media in order to characterize the suitability of our assay procedure for quantitative analyses of SEPP in this matrix. To this end, HepG2 cells were stimulated with 100 nM Na_2SeO_3, and the conditioned cell culture media were collected after 72 h to obtain sufficient amounts of SEPP for decay analyses. One set of samples was incubated at RT over different periods of time. A second independently generated set of samples was subjected to multiple freeze–thaw cycles. SEPP turned out to be highly stable at room temperature in conditioned HepG2 cell culture media within a time period of 24 h (Supplementary Figure S1). The average SEPP concentration was 0.13±0.02 mg SEPP/L at the starting point of incubation and changed within the following 24 h only within a range of ±0.02 mg SEPP/L. The freshly collected conditioned cell culture medium in the second set of samples had a starting SEPP concentration of 0.17 mg/L (Supplementary Figure S2). A single freeze–thaw cycle reduced SEPP concentrations significantly to 0.14 mg SEPP/L. The following four freeze–thaw cycles were of little effect only. After six freeze–thaw cycles, SEPP concentrations were reduced to 58% of the initial value. Similar stability of SEPP upon prolonged room temperature incubations and a comparable decline of SEPP concentrations upon a first freeze–thaw cycle were observed with conditioned media from Huh-7 cells (not shown).

3.4. Comparison of different selenocompounds to support SEPP production

The Se compounds tested elicited characteristic time- and dose-dependent effects on SEPP concentrations in the hepatic cell culture media. Sodium selenite (Na_2SeO_3) is already known as a very effective Se supplement readily supporting SEPP biosynthesis in liver cells as described earlier [16,42,43]. Compared to sodium selenate (Na_2SeO_4), it elicited a significantly stronger inducing effect on SEPP accumulation in a dose- and time-dependent manner (Fig. 2A). Comparing all the different selenocompounds under our conditions chosen, we found that 100 nM Na_2SeO_3 was the most effective and raised SEPP concentrations in the culture media to 0.125 mg SEPP/L after the maximal incubation period of 72 h. This constitutes a 6.3-fold increase in comparison to the PBS control (0.02 mg SEPP/L). Na_2SeO_4 addition yielded only a 2.5-fold induction of SEPP under these conditions (100 nM test substance, 72 h of incubation).

Comparison of the stereoisomers of SeMet, i.e., L-SeMet and an equimolar mixture of D-SeMet and L-SeMet, indicated that the L-isomer has higher bioavailability (Fig. 2B). Again, both preparations increased SEPP content in the conditioned media significantly compared to control in a time-dependent manner. Maximal effects were slightly different, and the pure L-isomer of SeMet increased SEPP production 1.2 times more than the isomer mixture under the optimal incubation conditions.

In comparison, the incubations with stereoisomers of selenocystine ($SeCys_2$) were more efficient and yielded comparable results independent of the isomer ratio (Fig. 2C). Maximal stimulation of SEPP production was 6.0 times higher compared to control, and final values were close to the maximal level obtained with Na_2SeO_3 as described above.

The glutathione derivative selenodiglutathione (GSSeSG) proved to be another strong stimulator of hepatic SEPP production. Maximal levels were again similar to the prototype substance Na_2SeO_3 and averaged at 6.3-fold over control values upon 100 nM of GSSeSG after 72 h (Fig. 2D). In comparison, ebselen proved stable under the incubation conditions chosen, and the extracellular SEPP concentrations were not significantly different from control.

Both methylseleninic acid (MeSeA) and selenomethylselenocysteine (SeMeSeCys) were less efficient than Na_2SeO_3, SeMet, $SeCys_2$ or GSSeSG in supporting SEPP biosynthesis and secretion. One hundred nanomolars of MeSeA increased extracellular SEPP concentrations after 72 h by twofold above control. In comparison, SeMeSeCys increased extracellular SEPP concentrations to five times of the control levels (Fig. 2E). Again, kinetics of extracellular SEPP accumulation was linear with incubation time indicating a well-chosen experimental set-up for the assessment of efficiency of the different selenocompounds.

Upon comparison of all the substances tested, the selenocompounds can be grouped into three main classes. Highly bioavailable and efficient supporters of hepatic SEPP production and secretion are the classical inorganic supplement Na_2SeO_3, as well as $SeCys_2$ independent of stereoisomer, GSSeSG and SeMeSeCys. Incubations with Na_2SeO_4, each of the SeMet isomers or MeSeA were also effective in increasing extracellular SEPP concentrations, albeit with a considerably reduced potency as compared to the first group of substances. Ebselen was the only stable selenocompound tested and did not affect extracellular SEPP accumulation to a significant extent (Fig. 2F).

Very similar results and the same final classification scheme were obtained with Huh-7 cells (data not shown). Again, ebselen turned out to be completely without effect. MeSeA, both SeMet isomers and Na_2SeO_4 were less efficient in supporting SEPP expression compared to the aforementioned highly bioavailable and efficient selenocompounds, i.e., Na_2SeO_3 and both $SeCys_2$ stereoisomers, GSSeSG and SeMeSeCys. Upon comparison of both cell lines, HepG2 cells synthesized on average four times more SEPP compared to Huh-7 cells, indicating their superior suitability for such studies.

3.5. Analysis of GPX1 expression as an intracellular biomarker of Se availability

GPX1 is a sensitive intracellular indicator of Se availability in hepatocytes and responds with reduced activity and reduced mRNA concentrations to a decline in hepatic Se status [12,16,42]. In order to compare the effects of the different selenocompounds on SEPP biosynthesis with an intracellular selenoenzyme, GPX1 activity assays were conducted with HepG2 cell homogenates after 72 h of incubation. A very similar picture as before was obtained with regard to the nutritional value of the different selenocompounds (Fig. 3). Again, Na_2SeO_3, $SeCys_2$, GSSeSG and SeMeSeCys turned out to serve as readily available sources of Se for increasing selenoprotein biosynthesis and inducing GPX1 activity by >2-fold under the experimental conditions. At the same time, Na_2SeO_4 and SeMet failed to show a significant induction, while ebselen and MeSeA were again without any detectable effect.

3.6. Assessment of hepatocyte-specific toxicity of the different selenocompounds

Certain selenocompounds are discussed as potential chemopreventive or chemotherapeutic agents partially based on their toxicity, inducing apoptosis preferentially in fast dividing tumour cells [44–48]. Identifying suitable selenocompounds which increase

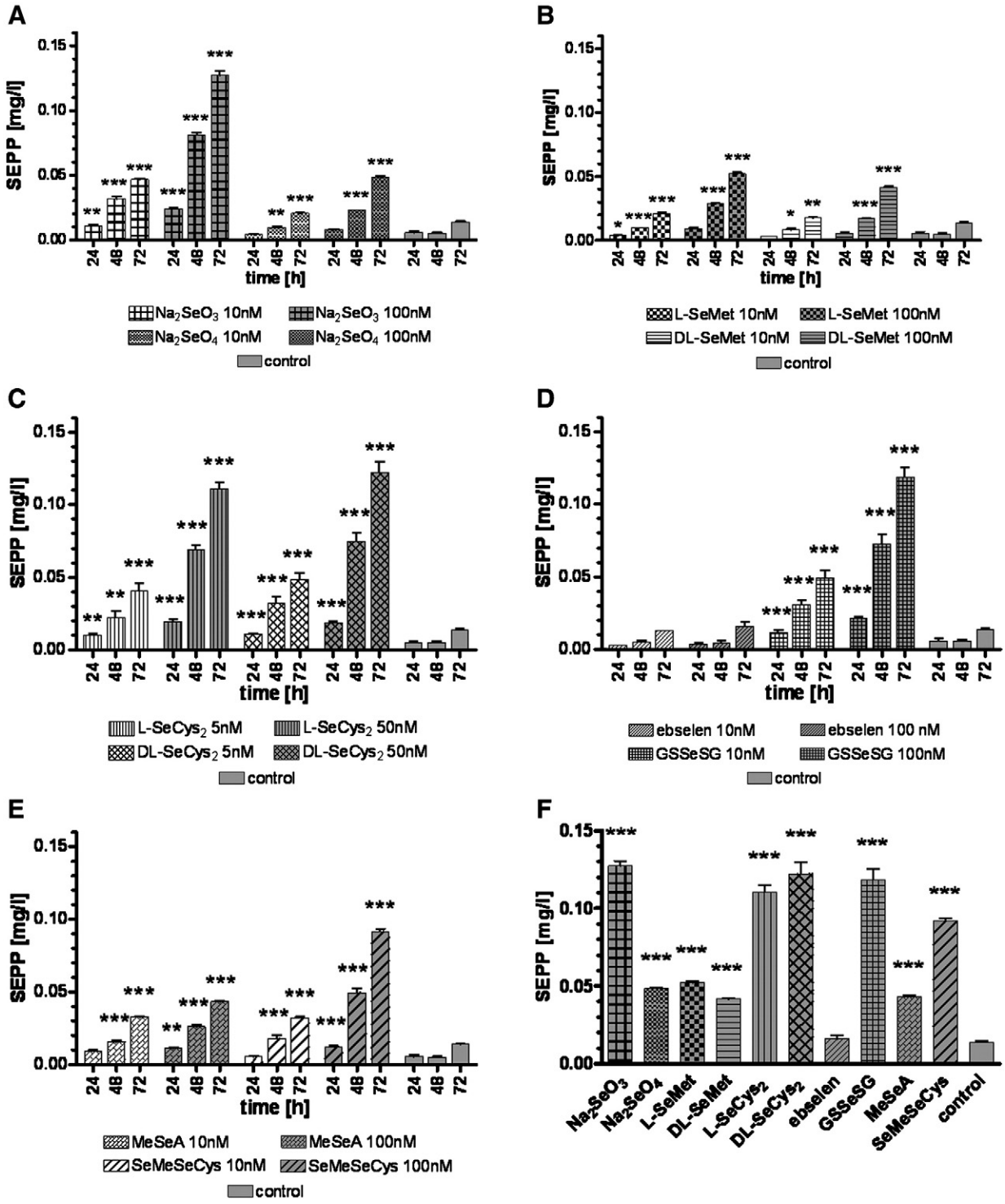

Fig. 2. Time- and dose-dependent effects of selenocompounds on SEPP production by HepG2 cells. HepG2 cells were incubated with different selenocompounds and concentrations (5 or 10 and 50 or 100 nM, respectively) for 24, 48 and 72 h, and conditioned cell culture media ($n=5$) were analysed by SEPP quantification assay. Data represent means±S.E.M. of duplicate measurements of three independent experiments. Statistical significance in relation to control was calculated using ANOVA and Dunnett's T3 test: *$P<.05$, **$P<.01$, ***$P<.001$. (A) Comparison of Na_2SeO_3 and Na_2SeO_4 at 10 and 100 nM after incubation for 24, 48 and 72 h. (B) Same type of analysis for L-SeMet and DL-SeMet. (C) Same type of analysis for L-$SeCys_2$ and DL-$SeCys_2$. (D) Same type of analysis for ebselen and GSSeSG. (E) Same type of analysis for MeSeA and SeMeSeCys. (F) Overview of the selenocompounds tested at 50 nM (L- and DL-$SeCys_2$) or 100 nM (all the other) and after 72 h of incubation.

hepatic SEPP production thus needs to be combined with studies on their toxicity. To this end, HepG2 cells were incubated with increasing concentrations of the different selenocompounds ranging from 0.1 nM to 1.0 mM and MTT tests were performed. Selenocompound-specific LD_{50} values for HepG2 cells were deduced after three different periods of time, i.e., after 24, 48 and 72 h of incubation. This type of time-resolved cell death analysis ensures reproducibility of the data and indicates potential time-dependent mechanisms.

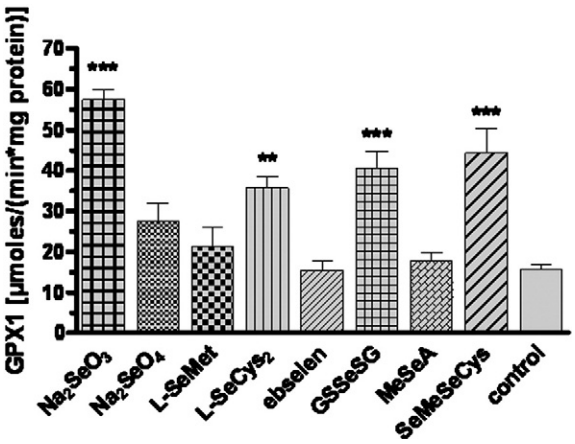

Fig. 3. Effects of the selenocompounds on GPX activity in HepG2 cells. GPX1 activity was determined by a coupled photometric test using *t*-butylhydroperoxide as substrate. HepG2 cells were stimulated for 72 h with the different selenocompounds (50 or 100 nM) as described before. Data represent mean±S.E.M. of two independent experiments in duplicate measurements. Statistical significance in relation to control was calculated using ANOVA and Dunnett's test: $^{**}P<.01$, $^{***}P<.001$.

Most selenocompounds were toxic at high concentrations with the exception of ebselen and both SeMet stereoisomers. Comparing the remainder of the selenocompounds, it was found that their effect curves and deduced LD_{50} values differed to some extent (Table 1). Upon comparison of the two inorganic Se compounds, Na_2SeO_3 was found to be more toxic and decreased viability was already observed in the micromolar concentration range (Supplementary Figure S3a). Upon 72 h of incubation, an LD_{50} of 2 µM was determined for Na_2SeO_3 which is >30 times lower than the corresponding value of Na_2SeO_4 (Supplementary Figure S3b). The same tendencies were observed at earlier time points highlighting the strongly different toxicities of selenite *vs*. selenate. Moreover, toxicity of Na_2SeO_3 showed a characteristic time dependence (Table 1). Comparing the two different $SeCys_2$ preparations, it was found that the mixture of the isomers (Supplementary Figures S3c) was better tolerated than the pure L-isomer (Supplementary Figure S3d) and displayed a relatively flat toxicity curve. In comparison, L-$SeCys_2$ and GSSeSG (Fig. 4A) were relatively toxic and only few cells survived a 10-µM concentration for 72 h. SeMeSeCys was tolerated well and toxic effects were only observed at concentrations exceeding 100 µM (Fig. 4B). The time-dependent analyses indicate that the toxic effects of the selenocompounds were relatively fast. This finding implies that their toxicities are rather a direct effect and not secondary to changing gene transcription patterns or *via* slowly accumulating damage in critical cellular components. The peak concentrations of the selenocompounds appear to determine the outcome either by directly interfering with survival-controlling pathways or by being converted fast to toxic metabolites. In summary, the LD_{50} values determined were compound-specific data and ranged over two orders of magnitude depending on the selenocompound applied.

Table 1
Summary of the time-resolved LD_{50} values determined by the MTT test in HepG2 cells

Toxicity	Se compound	LD_{50}, µM (mg/L)		
		24 h	48 h	72 h
High	MeSeA	0.53 (0.07)	0.59 (0.07)	0.20 (0.03)
	L-$SeCys_2$	1.1 (0.3)	0.2 (0.06)	1.7 (0.3)
Moderate	Na_2SeO_3	11 (1.9)	5.5 (0.9)	1.9 (0.3)
	DL-$SeCys_2$	14 (4.7)	13 (4.5)	6.7 (2.3)
	GSSeSG	7.5 (4.9)	2.8 (2.1)	2.1 (1.4)
Low	Na_2SeO_4	124 (23.4)	134 (25.3)	67 (12.6)
	SeMeSeCys	235 (42.9)	164 (29.9)	177 (32.2)
No	Ebselen	–	–	–
	L-SeMet	–	–	–
	DL-SeMet	–	–	–

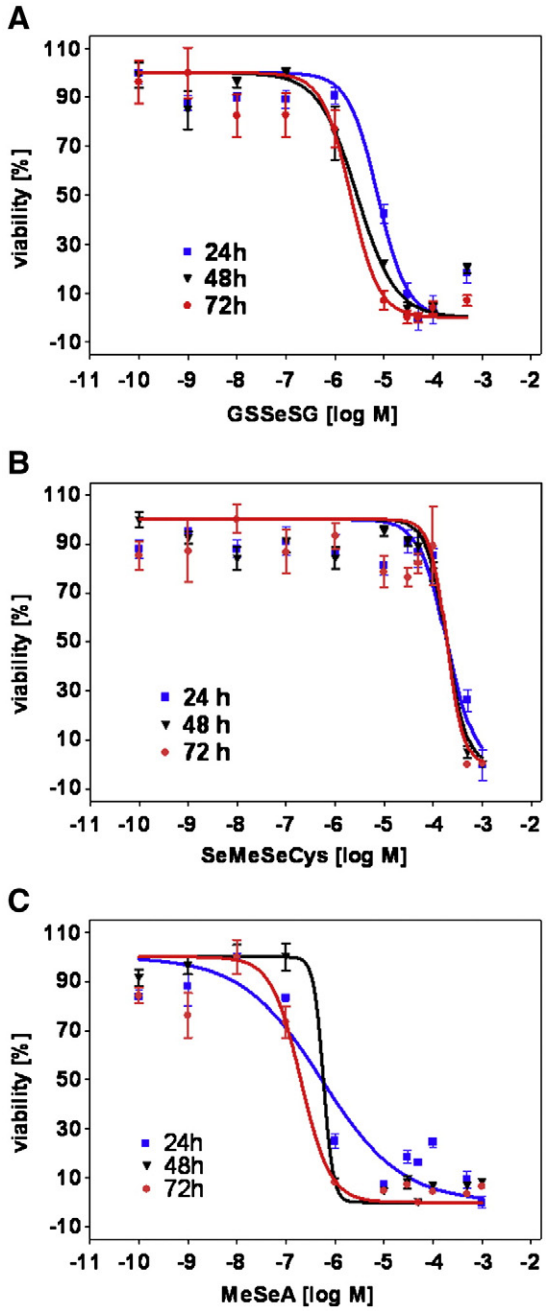

Fig. 4. Toxicity of different selenocompounds in a concentration range of 0.1 nM to 1.0 mM after 24 h (■), 48 h (▼) or 72 h (●) of incubation in HepG2 cells as determined by MTT test. Concentration–response curves and LD_{50} values were calculated using GraphPad Prism 4. Data represent means±S.E.M. in quadruplicate measurements of (A) GSSeSG, (B) SeMeSeCys and (C) MeSeA.

As mentioned above, ebselen and both SeMet isomers were not toxic under these conditions as judged by the MTT test with HepG2 cells. No LD_{50} values could thus be determined for these selenocompounds. In contrast, MeSeA was the most toxic of the substances tested (Fig. 4C). Accordingly, four categories of selenocompounds can be discriminated, i.e., highly, moderately, slightly and non-toxic substances, respectively (Table 1). These substance-specific charac-

teristics need to be combined with the nutritional value of the different selenocompounds as determined above. A more complete picture emerges from the data clearly indicating that the most important physiological qualities do not correlate (Fig. 7).

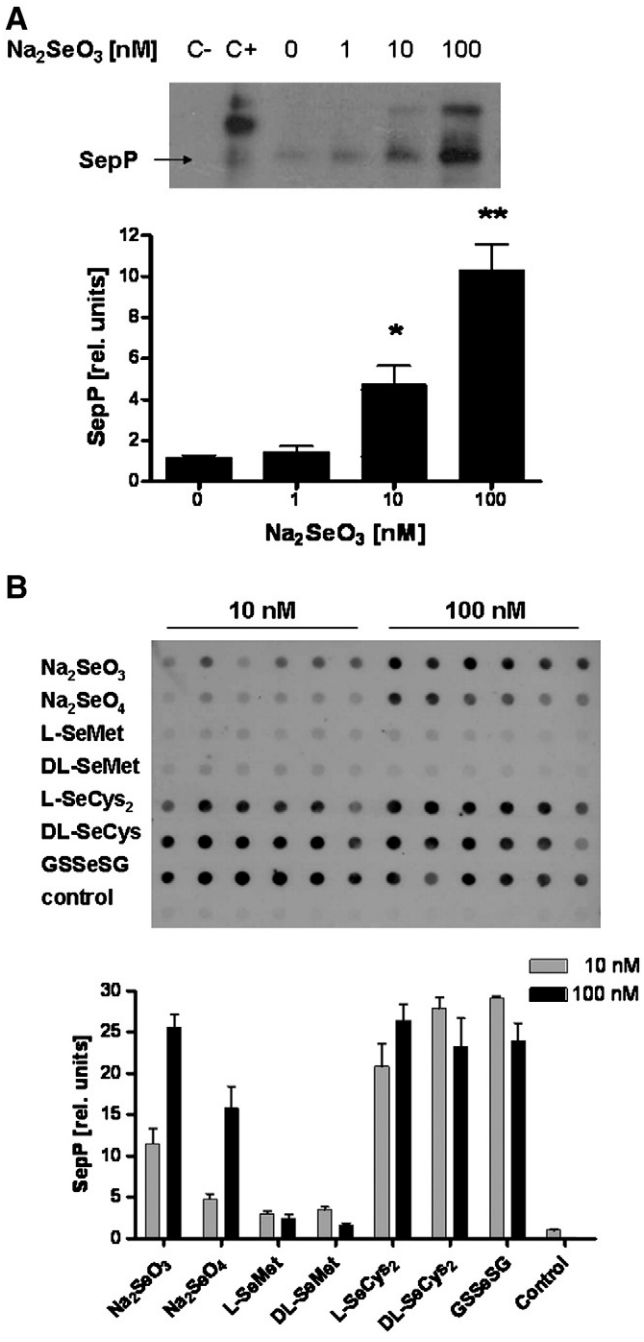

Fig. 5. Test for species specificity of the effects observed in human hepatocytes by analysing murine Hepa 1–6 cells. (A) Western blot analysis of Na_2SeO_3-mediated stimulation of SepP production. Hepa 1–6 cells were stimulated with Na_2SeO_3 (0, 1, 10 and 100 nM, respectively) for 72 h. Increasing SepP amounts were quantified after protein precipitation in conditioned media by Western blot analysis. Serum samples of wild-type (C+) and SepP-KO (C−) mice served as positive and negative controls, respectively. Statistical significance in relation to control ($n=3$) was calculated using ANOVA and Dunnett's T3 test: *$P<.05$, **$P<.01$. (B) Dot blot-based comparison of different selenocompounds increasing SepP production. Hepa 1–6 cells were stimulated with different selenocompounds (10 and 100 nM, respectively) for 72 h, and SepP concentrations were analysed in conditioned cell culture media. Na_2SeO_3, GSSeSG, and L- and DL-$SeCys_2$ were the most effective in increasing SepP concentrations, while L- and DL-SeMet were relatively ineffective.

3.7. Comparison of human and murine hepatocytes

In order to examine whether the effects of the different Se compounds on SEPP production in HepG2 and Huh-7 cells were human specific, the murine hepatocarcinoma cell line Hepa 1–6 was included into the analyses. The amount of secreted SepP into the conditioned cell culture media was detected and quantified after acetone precipitation and Western blot analyses since the luminometric assay used above is specific for human SEPP. Serum of wild-type and homozygous SepP knockout mice served as positive and negative controls, respectively. The typical pattern of immunoreactive SepP in murine serum was detected in wild-type but not in knockout mice, verifying the specificity of the antibodies used and the suitability of the Western blot analysis procedure (Fig. 5A). As observed before with human hepatocytes, SepP secretion into conditioned cell culture medium was dose-dependently increased in Hepa 1–6 cells by the prototypic stimulator Na_2SeO_3 (Fig. 5A). A dot blot protocol was developed for murine SepP quantification to enable the comparison of multiple samples simultaneously. Blotting parameters and antibody concentrations were titrated in order to improve signal-to-noise ratio using serum from wild-type and knockout mice as test samples. Accordingly, aliquots of conditioned cell culture media of Hepa 1–6 cells incubated for 72 h in the presence of 10 or 100 nM of the selenocompounds were directly applied onto a nitrocellulose membrane. Incubation with Na_2SeO_3 yielded the same dose-dependent increase in extracellular SepP concentrations by dot blot analysis (Fig. 5B) as observed before with the Western blots (Fig. 5A). In agreement with the results in human HepG2 and Huh-7 cells, Na_2SeO_3 was more efficient than Na_2SeO_4 in supporting SepP biosynthesis. GSSeSG, L-$SeCys_2$ and the DL-$SeCys_2$ isomers were strong stimulators of SepP biosynthesis, even though the concentration-dependent effects appeared less well pronounced than before with the human cells. A species-specific difference was observed for L- and DL-SeMet which were moderately effective in human cells but did not support SepP biosynthesis in murine Hepa 1–6 cells after incubation periods of 72 h in the concentration range of 10–100 nM.

4. Discussion

Se is an important micronutrient in the human diet and numerous health effects have been associated with differences in Se intake and Se status [8,9,49]. In general, Se intake and status differ considerably among the different countries due to geographical differences, i.e., largely varying Se concentrations in agricultural soils [50]. Se is taken by many individuals as a promising health-promoting micronutrient supplement largely without controlling total intake and accumulating blood levels [32]. This type of self-medication appears not without risk as indications of adverse health effects on insulin sensitivity, diabetes or cardiovascular risk have recently been deduced from the analysis of US individuals with high Se status and intake, respectively [51–53]. In addition, there are regular reports on wrongly calculated and mixed supplements which enter the market and pose a health risk to the users [54]. Accordingly, a deeper knowledge on the metabolic differences of the various selenocompounds with regard to their nutritional value vs. potentially dangerous side effects is definitely needed [30,55], even though the main metabolic pathways are well characterized (Fig. 6).

In human trials, both Na_2SeO_3 and SeMet have proven effective and increased serum SEPP concentrations in poorly supplied [56] but not in well-supplied individuals [29]. Similarly, metabolism of different selenocompounds was compared in Se-deficient Wistar rats with isotope-labelled substrates. Bioactivity in terms of increasing SepP serum concentrations decreased in the order of Na_2SeO_3 to SeMeSeCys to SeMet [57]. These *in vivo* results are in line with our findings and support the notion that hepatocytes in culture can serve

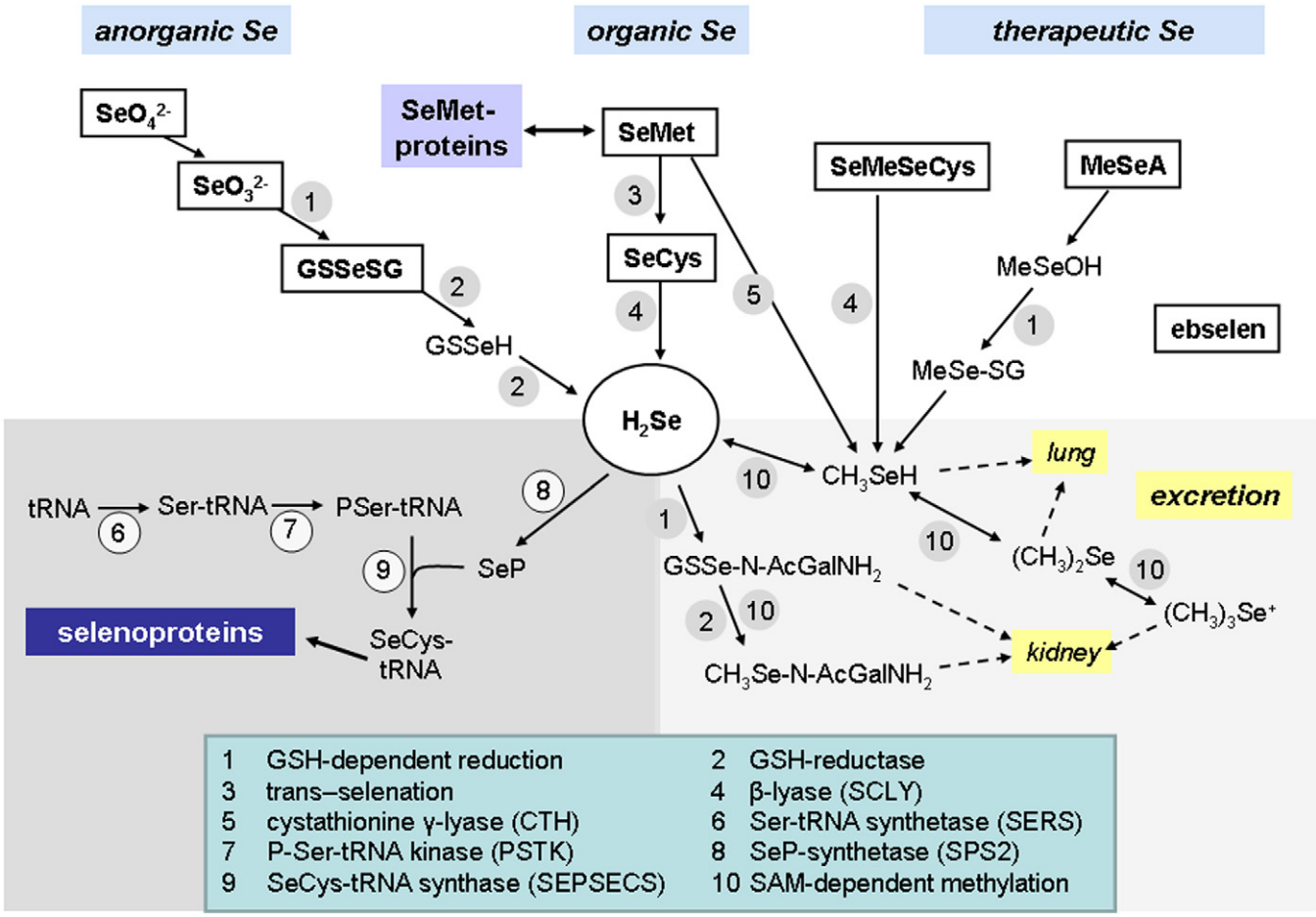

Fig. 6. Schematic overview of the metabolism of the different selenocompounds. Se is biologically active in a variety of natural and synthetic forms, but their metabolic pathways differ. SeMet is mainly found in plants and can be incorporated unspecifically into SeMet-containing proteins or transselenated to SeCys, which is then converted into selenide (H_2Se). Alternatively, methylselenol (CH_3SeH) can be liberated from SeMet by γ-lyase and can be further converted to H_2Se for anabolic purposes or excretion. Similarly, sodium selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) undergo a reductive metabolism yielding H_2Se as a central intermediate. Ebselen is a stable glutathione peroxidase mimetic which is not metabolized under normal circumstances. SeMeSeCys and MeSeA can directly serve as precursors of CH_3SeH. Excessive Se is detoxified by methylation *via* *S*-adenosylmethionine (SAM) yielding dimethylselenide ($(CH_3)_2Se$) or trimethylselenonium ($(CH_3)_3Se^+$) for excretion *via* the lungs and kidneys, respectively. Alternatively, selenosugars are formed under physiological conditions in liver and excreted *via* the urine. Synthesis of SeCys-containing selenoproteins depends on loading a serine residue onto $tRNA^{[Ser]Sec}$ by seryl-tRNA synthetase (SerS). Seryl-$tRNA^{[Ser]Sec}$ is then phosphorylated and activated by phosphoseryl-tRNA kinase (PSTK). Similarly, H_2Se is phosphorylated by selenophosphate synthetase 2 (SPS2). Selenocysteine synthase (SEPSECS) finally fuses these two energy-rich substrates to yield Sec-$tRNA^{[Ser]Sec}$ as a limiting and eponymous component for selenoprotein biosynthesis.

as a valuable tool for analysing and comparing the different selenocompounds. Accordingly, the value of selenite as a readily available selenocompound for selenoprotein biosynthesis had already been highlighted in hepatocyte before [43]. The prime role of liver for regular Se metabolism has been corroborated by a number of complementary approaches, e.g., by analyzing patients with hepatitis or cirrhosis [58,59], by applying isotope-labelled selenocompounds followed by time-resolved analysis of circulating Se-containing proteins [57,60], or by transgenic mouse models with liver-specific knockout of selenoprotein-specific tRNA [33,61] or hepatocyte-specific overexpression of *SEPP* [21]. The main source of Se in the human diet is organic Se in the form of SeMet, SeCys and derivatives thereof, mainly as integral constituents of animal and plant proteins [2,30]. Other Se-containing compounds are normally not found in nutritionally relevant amounts. These selenocompounds proved of little toxicity in our analyses except for the L-SeCys isomer which is unlikely to reach the hepatocyte *in vivo*.

Selenocompounds that can be converted into monomethylated Se, e.g., SeMeSeCys or MeSeA, are considered as promising agents in cancer prevention and treatment [44,62]. From our analysis, these two selenocompounds elicit very different activity profiles; MeSeA is highly toxic and of little nutritional value, while SeMeSeCys is a well-tolerated and readily metabolized Se source. Especially the comparison of these two paradigmatic substances evokes the question on the rationale for a supplementation effort in the context of cancer prevention and treatment, i.e., whether a safe and likely preventive correction of a Se deficit is intended or a targeted destruction of sensitive cancer cells is aimed for. Clearly, these two selenocompounds differ strongly in their cellular effects, irrespective of their final metabolism to monomethylselenide, and thus their clinical use needs to be clearly defined and allocated to specific and limited purposes.

Despite these differences, the chemopreventive net effect of chemically different selenocompounds might still be similar in a living organism; both small selenocompounds and selenoproteins contributed to the chemopreventive activity of Se in a mouse model of colon tumorigenesis [63]. Similarly, very low and exceedingly high dietary Se concentrations were chemopreventive in a mouse model of hepatocarcinogenesis [64]. In this study, the molecular mechanisms were likely very different; selenoprotein biosynthesis is only affected at low to adequate Se supply but not at exceedingly high supraphysiological concentrations, indicating that selenocompound-specific alternative mechanisms were elicited independent of

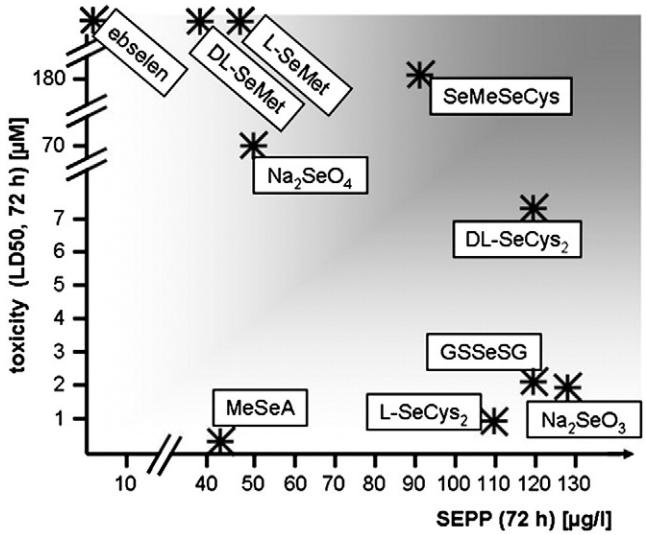

Fig. 7. Overview of the relative toxicities and nutritional values of the selenocompounds tested. Toxicity and SEPP production are indicated 72 h after incubating HepG2 cells with the respective selenocompounds. This picture highlights that there is no correlation between these two most important physiological qualities of the selenocompounds.

selenoprotein biosynthesis [64]. Moreover, another recent trial in a rat model of hepatocarcinogenesis highlighted that protective effects of Se can be elicited both before and after the chemical induction of liver cancer, indicating multiple modes of action, i.e., both chemopreventive and anticarcinogenic activities [65]. In another recent proteomic comparison, both SeMeSeCys and Na_2SeO_4 increased Se status but only SeMeSeCys affected the hepatic biosynthesis of two other physiologically important serum proteins, i.e., apolipoprotein E and transthyretin [66]. Together, these studies indicate the complex and multifaceted metabolic and physiologic outcome when hepatocytes are exposed to different selenocompounds.

Certain selenocompounds like ebselen are already used in clinics to treat, e.g., ischemic stroke or aneurysmal subarachnoid haemorrhage as antioxidative and anti-inflammatory drugs [67,68]. Notably, ebselen is known to act catalytically without liberating its Se moiety, which remains part of the ring structure even upon being exposed to biological systems [69]. These qualities are convincingly verified in our analyses as even high concentrations of ebselen were without effect on SEPP production, GPX1 activity or cell survival. Inorganic Se in the forms of Na_2SeO_3 and Na_2SeO_4 does not represent natural food constituents but is in widespread use as nutritional supplements in certain food stuffs, during individually conducted supplementation efforts or within experimental or clinical trials [70–73]. Usually, Na_2SeO_3 is the preferred choice when fast supplementation effects are desired as, e.g., in the case of adjuvant treatment of Se-deficient sepsis patients in intensive care units [74]. Its great value with respect to increasing hepatic GPX1 activity and improving hepatic SEPP biosynthesis is corroborated by our results, yet its relatively high toxicity compared to Na_2SeO_4 and the almost equally effective SeMeSeCys raises some doubt on its first choice in light of the relatively high dosages applied in clinics. Probably, SeMeSeCys would be better tolerated by a stressed and diseased liver in order to normalize Se metabolism and SEPP production during critical illness [75].

Collectively, our results clearly indicate that bioavailability and toxicity are not correlated but rather represent a selenocompound-specific quality which becomes very obvious when both qualities are plotted in a single diagram (Fig. 7). It is still too often in nutrition research and basic science alike that the different selenocompounds are collectively denoted as "selenium" without specifying their chemical form and potential side-effects. This is most strikingly exemplified by current reference values for Se intake in which the chemical element is given but the character of the respective compound is ignored (e.g., TDI, RDA, LOEL or NOAEL values).

This is an unfortunate situation as the differential toxicities elicited by selenocompounds definitely need to be taken into account when *in vivo* supplementation studies are intended. Na_2SeO_3, Na_2SeO_4 and SeMet serve as well-characterized examples, and their toxicity profiles reported herein are very different and in good agreement with the findings in a recent study comparing primary and transformed hepatocytes [76]. In general, Na_2SeO_3 proves more toxic than Na_2SeO_4, while SeMet is usually well tolerated. The uptake and toxicity of Na_2SeO_3 have been shown to depend on extracellular reduction and a cystine gradient [77]. Its high toxicity has been verified in different species and systems, e.g., with rodent embryos [78], with prostate cells in culture [79] or with hepatocytes from rainbow trout [80]. Our experiments extend these studies by categorizing this property in comparison to the other selenocompounds into classes of toxicity represented by these paradigmatic substances. Notably, SeMeSeCys was confirmed as being of relatively little toxicity among all the selenocompounds while still being highly bioavailable [81].

Since the positive effects of the different selenocompounds on GPX1 activity and SEPP production did not correlate to their toxicities but rather were molecule-specific qualities, the clinical preference for SeMet, Se-enriched yeast or Na_2SeO_3 may be reconsidered with respect to the intention of supplementation. Chemoprevention might best be achieved by selenocompounds causing apoptosis preferentially in cancerous cells [82] or inducing a ROS-dependent senescence response especially in noncancerous cells [83]. In this respect, selenocompounds being readily converted to methyl–selenol have proven effective in certain animal tumour models [84]. In contrast, other less toxic selenocompounds may be better suited when avoidance of Se deficiency, support and replenishment of sepsis patients or treatment of autoimmune thyroid disease is intended [9]. In these applications, Na_2SeO_3 has usually proven very effective, potentially due to its high bioavailability and fast supplementation effects [74,85,86]. From our studies, we can conclude that there are alternative Se-containing organic compounds that might be equal or superior to SeMet or Na_2SeO_3 as Se supplements and therefore should be taken into consideration for future supplementation studies in animals and humans. In this respect, SeMeSeCys qualified as a nontoxic and highly bioactive selenocompound which might prove as an advantageous supplementation form of Se under conditions where toxicity is to be avoided.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.08.006.

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