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RESEARCH ARTICLE

Cytotoxic activity of selenosulfate versus selenite in tumor cells depends on cell line and presence of amino acids

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Abstract Based on acute cytotoxicity studies, selenosulfate ($SeSO_3^{-}$) has been suggested to possess a generally higher toxic activity in tumor cells than selenite. The reason for this difference in cytotoxic activity remained unclear. In the present study, cytotoxicity tests with human hepatoma (HepG2), malignant melanoma (A375), and urinary bladder carcinoma cells (T24) showed that the selenosulfate toxicity was very similar between all three tested cell lines (IC_{50} 6.6–7.1 μM after 24 h). It was largely independent of exposure time and presence or absence of amino acids. What changed, however, was the toxicity of selenite, which was lower than that of selenosulfate only for HepG2 cells ($IC_{50} > 15$ μM), but similar to and higher than that of selenosulfate for A375 (IC_{50} 4.7 μM) and T24 cells (IC_{50} 3.5 μM), respectively. Addition of amino acids to T24 cell growth medium downregulated short-term selenite uptake (1.5 versus 12.9 ng Se/10^6 cells) and decreased its cytotoxicity (IC_{50} 8.4 μM), rendering it less toxic than selenosulfate. The suggested mechanism is a stronger expression of the x_c^- transport system in the more sensitive T24 compared to HepG2 cells which creates a reductive extracellular microenvironment and facilitates selenite uptake by reduction. Selenosulfate is already reduced and so less affected. The cytotoxic activity of selenosulfate and selenite to tumor cells therefore depends on the sensitivity of each cell line, supplements like amino acids as well as the reductive state of the extracellular environment.

Keywords HepG2 · T24 · A375 · Non-essential amino acids · Cellular selenium uptake · L-glutamic acid

Introduction

Selenium (Se) is an essential trace element for humans. Various selenium compounds are known to possess anticarcinogenic properties, e.g., selenite (Na_2SeO_3) is often used in clinical trials as addition to commonly applied anticancer chemotherapy drugs (Dennert and Horneber 2006). High concentrations of the anticancer drug cisplatin can result in different side effects such as leukocyte reduction, diarrhea, vomiting, edema, or nephrotoxicity. As an essential antioxidant selenite can reduce the toxicity of cisplatin without compromising its anticarcinogenic activity as shown in animal studies (Baldew et al. 1989; Camargo et al. 2001; Markovic et al. 2011). However, high doses of selenite have been shown to exert strong cytotoxic effects themselves, e.g., on prostate (Li et al. 2007), ovarian (Park et al. 2012a), liver (Shen et al. 1999), or lung cells (Park et al. 2012b). Due to the narrow concentration range between beneficial and toxic effects and a potential negative influence also on healthy tissue, application of selenite in cancer treatment is still discussed quite controversially (Micke et al. 2009; Vinceti et al. 2001; Zhang et al. 2008b).

A recent patent (Patent US 2010/0172822) claims selenosulfate (Na_2SeSO_3) to be preferable over selenite and

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suggests its use as complementary substance in anticancer therapy (Zhang 2010). Mice studies showed that selenosulfate effectively reduced gastrointestinal toxic effects induced by cisplatin from 80 to 6 % (Li et al. 2012) without disturbing its therapeutic effect on tumor cells (Zhang et al. 2008b). In addition, while long-term (55 days) administration of selenite resulted in dose-dependent growth suppression and hepatoxicity in mice, selenosulfate administration in the same concentrations (12.7 and 19 μM) did not result in toxic symptoms (Li et al. 2012). That means while being as efficient as selenite in reducing negative side effects of chemotherapy drugs such as cisplatin, selenosulfate is less toxic to healthy tissue, thus, higher doses can be applied.

An interesting side effect in selenite-based cancer therapy is that cell culture studies suggest selenite to have a higher cytotoxicity in tumor cells compared to healthy cells, shown e.g., in studies on human malignant glioma (Kim et al. 2007) and osteosarcoma cells (Chen et al. 2012) versus comparable healthy cells. The differences in toxicity for tumor versus healthy cells are mainly explained by different uptake mechanisms, which in turn are governed by the extracellular reductive capacity (Olm et al. 2009). The redox state of the extracellular environment largely depends on the cysteine/glutamate exchange system (x_c^-) first identified by Bannai et al. (1986) and the presence of multidrug resistance proteins (MRP) which are overexpressed in many tumor cell lines (reviewed in Conrad and Sato 2012). The x_c^--overexpression drives the intracellular cystin/cysteine redox cycle. Cysteine is then increasingly secreted by MRP into the extracellular environment and induces increased selenium uptake by formation of more reduced selenium compounds, especially HSe^-. Both HSe^- and selenite enter the cells by anion channels, but there is a higher affinity for HSe^- (Conrad and Sato 2012). A strong relationship was found between extracellular thiol production, selenite uptake, and cellular susceptibility to selenite in different lung tumor cells (Olm et al. 2009). Selenite uptake was increased by extracellular reduction through e.g., GSH addition and decreased by extracellular oxidation (through addition of 55′-dithiobis-(2-nitrobenzoic acid)) (Olm et al. 2009). Addition of extracellular thiols also increased selenite uptake in a keratinocyte model (Ganyc &Self 2008). Amino acids might decrease selenite uptake, at least the reverse process of selenite decreasing the uptake of amino acids was confirmed previously (Hogberg and Kristoferson 1979; Vernie et al. 1974). An influence of amino acids on selenite uptake actually generates a problem when comparing cytotoxicity data of different cell culture studies. Numerous different cell growth media compositions have been used for growing the same cell line and non-essential amino acids (NEAA) have been added in some studies (McKelvey et al. 2015; Zhang et al. 2008a) but not in others (Chu and Crawford-Brown 2006; Peng et al. 2007; Zou et al. 2007) without separating pure selenite from combined selenite-NEAA effects.

For selenosulfate, patent US 2010/017282 claims that it has an even higher activity than selenite to suppress and kill specifically tumor cells (Zhang 2010) which would be another advantage of its preferred application. The claim is based on acute cytotoxicity tests in cell culture studies with human hepatocellular carcinoma (HepG2), epithelial colorectal adenocarcinoma (Caco-2), and three different kinds of leukemia cells (HL60, T lymph adenoma, Daudi) (Zhang et al. 2008a). The amount of intracellular uptake or the effect of extracellular compounds on cytotoxicity and uptake were not investigated and no reason was given as to what could have caused the difference in cytotoxic activity between selenosulfate and selenite.

The goal of the present study was therefore to repeat the previously published cytotoxicity tests (Zhang et al. 2008a) to see if this claim can really be generalized to different types of tumor cells and to elucidate why selenosulfate should be more toxic than selenite. Besides human hepatoma cells (HepG2), which were selected for comparison with the previous studies (Zhang 2010; Zhang et al. 2008a), we tested two further tumor cell lines: malignant melanoma (A375) and urinary bladder carcinoma cells (T24). The T24 cells were selected because high selenium levels in the body have been shown to be inversely correlated with bladder cancer risk (Kellen et al. 2006) and selenium is discussed to act as a chemopreventive agent against bladder cancer (Brinkman et al. 2006). The A375 cells were selected because previous experiments related the presence of different selenium compounds — selenium nanoparticles (Chen et al. 2008), selenocystine (Chen and Wong 2008), and 1,4-Diselenophene-1,4-diketone (Vinceti et al. 2014) — to oxidative stress and mitochondrial dysfunction leading to apoptosis in A375 cells. Furthermore, selenium compounds were found to be able to enhance X-ray induced growth inhibition in A375 cells (Lo et al. 2008).

Using these three different cell lines, we compared cytotoxicity of selenosulfate and selenite for three different incubation times (24, 48, and 72 h) and, for HepG2 and T24 cells, in the presence and absence of NEAA as medium supplement. We also determined intracellular selenium concentrations to investigate the relation between uptake and respective selenium cytotoxicity.

Materials and methods

Synthesis and stability of selenosulfate standards

Selenosulfate was synthesized according to a published procedure (Zhang et al. 2008a). Briefly, selenite (Fluka Analytical, Steinheim, Germany), glutathione (GSH, Applichem, Darmstadt, Germany), and sulfite (Sigma-Aldrich, Steinheim, Germany) were mixed in a molar ratio of 1:4:4 and gently homogenized until the solution was clear.

The purity of the standard (91.7 ± 9.9 %, rest on average 6.8 % selenite and 1.5 % selenate) was determined by speciation analysis using anion exchange chromatography (Dionex) with an AS16 column, a 0.02–0.1 M NaOH-eluent and no anion suppressor, coupled to an inductively coupled plasma mass spectrometry (AEC-ICP-MS, XSeries2, Thermo-Fisher). The method had previously been described for separation of thioarsenates and thioantimonates (Planer-Friedrich et al. 2007). In the experimental solutions themselves we could not determine selenium speciation chromatographically due to interferences from the applied cell growth medium. Pretests under cell cultivation conditions (37 ° C, 5 % CO_2) showed after 72 h no precipitation of elemental red selenium which had previously been taken as an indication of selenosulfate instability (Zhang et al. 2008a). We therefore conclude that selenosulfate was stable during all our experiments.

Cell cultures

Both HepG2 and T24 cells were kindly provided by Prof. Dr. E. Dopp (Institute of Hygiene and Occupational Medicine, University Hospital Essen, Germany). The T24 cell line was primarily assumed to be the UROtsa cell line which is a non-tumorigenic urothelial cell line. However, several stocks of UROtsa cells were cross-contaminated and were recently identified as bladder tumor cell line T24 (Johnen et al. 2013). The A375 cells were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany). The HepG2 cells were grown in minimum essential medium with Earle's Salts (MEM, c·c·pro, Oberdorla, Germany) supplemented with 10 % FBS, 0.5 % gentamycine, 1 % L-glutamine, 1 % NEAA, and 1 % sodium pyruvate (all c·c·pro, Oberdorla, Germany). The T24 cells were cultivated in MEM supplemented with 10 % fetal bovine serum (FBS, Gibco), 0.5 % gentamycine (c·c·pro, Oberdorla, Germany), and 1 % L-glutamine (c·c·pro, Oberdorla, Germany). The A375 cells were cultivated in Dulbecco's modified Eagle Medium (DMEM, c·c·pro, Oberdorla, Germany) supplemented with 10 % FBS, 1 % L-glutamine, and 1 % penicillin-streptomycin (DMEM, c·c·pro, Oberdorla, Germany). All cell lines were incubated in a humidified atmosphere at 37 °C and 5 % CO_2 (Incubator Galaxy 170 S, New Brunswick Scientific) and passaged thrice weekly using trypsin (0.25 %, c·c·pro, Oberdorla, Germany) for T24 cells and trypsin-EDTA (0.25 %, c·c·pro, Oberdorla, Germany) for HepG2 and A375 cells. Before conducting toxicity experiments with the three cell lines, the thawed cells (HepG2 passage number P22, T24 passage number P28, A375 passage number P25) were passaged three times. To maintain comparable conditions, no more than ten further passages were performed with one cell line.

Non-essential amino acids were only used for standard cultivation of HepG2 cells. To investigate the effect of NEAA on selenosulfate and selenite cytotoxicity and uptake, experiments with HepG2 cells were additionally conducted in MEM without NEAA addition and with T24 cells in MEM with NEAA supplementation. To let the cells adapt to the modified medium, they were passaged for at least three times before starting an experiment.

MTT assay

For cytotoxicity testing, 5000 cells/well were allowed to attach for 24 h in 96-well plates (Falcon, Becton Dickinson, Meylan Cedex, France) and subsequently exposed to selenosulfate (0.17–25 μM) or selenite (0.1–15 μM). For each experiment, selenosulfate and selenite standards were prepared freshly, sterile-filtered, and diluted in autoclaved water (Ampuwa, Fresenius Kabi, Bad Homburg, Germany). All experiments were conducted as triplicate with six equally treated wells per replicate ($n = 18$). For HepG2 cells, the 24 h incubation experiments were conducted twice (each setup conducted in triplicate). In each experiment, six wells were treated with the same concentration. After the respective incubation time (24, 48, and 72 h for HepG2 and T24 cells, 24 h for A375 cells), cells were incubated for 2 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA). Afterwards, the formazan crystals were dissolved in a solubilization solution (10 g sodium dodecyl sulfate (Sigma-Aldrich, Steinheim, Germany) dissolved in 99.4 mL dimethyl sulfoxyde (Sigma-Aldrich, Steinheim, Germany) and 0.6 mL acetic acid (VWR PROLAB, Briare, France)), and the formazan product was spectrophotometrically measured at 570 nm (reference wavelength 630 nm) (Infinite 200 PRO, TECAN). Cell viability was calculated by setting the cell viabilities of non-treated cells to 100 %. Calculation of the substance's inhibitory concentration inducing 50 % cell viability (IC_{50}) was conducted by fitting an erfc(x) function to the measured cell viability values as published previously (Hinrichsen et al. 2014). The lower the IC_{50} value, the more toxic the substance.

Determination of intracellular Se

A total of 5×10^6 cells were incubated for 24 h with fresh growth medium containing 1 μM selenite or 1.7 μM selenosulfate, respectively. Both selenium compounds were freshly prepared. These concentrations were selected because they were shown to be non-cytotoxic after 24 h incubation (CV 98.8 ± 3.7 % and 94.9 ± 5.1 %, respectively). The exact selenium concentrations in selenosulfate and selenite solutions were analyzed by ICP-MS. For better comparability, results are reported in ng Se taken up per 10^6 cells normalized to μM of initially applied selenium. The medium was removed after 24 h and the cells were successively rinsed with Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO),

Ampuwa, and 0.1 mM 2,3-dimercapto-1-propanesulfonic acid sodium salt monohydrate (DMPS, Alfa Aesar, Karlsruhe, Germany, purity 95 %) according to a published procedure (Hippler et al. 2011) to ensure the absence of extracellular selenium. Cells were trypsinized, collected in 2 mL DPBS, and mechanically lysed using glass beads (Retsch, Haan, Germany). Selenium uptake after 24 h incubation was additionally quantified in HepG2 cells grown in NEAA-free medium and in T24 cells grown in medium supplemented with NEAA. Total selenium concentrations in the cell lysates were determined without any further dilution by ICP-MS.

Statistical analyses

Separate two-way analyses of variance (ANOVA) were used to test for significant differences in the effects of incubation time and NEAA addition on cell viability curves of the two different selenium species. All data met assumptions of homogeneity of variance and normal distribution. Statistical analyses were performed with SPSS version 21.0 (IBM, Armonk, New York, USA).

Results

Comparing the three investigated cell lines - HepG2, A375, and T24 cells -, it becomes obvious that only HepG2 cells showed the expected trend of selenosulfate being more toxic than selenite (Fig. 1). The IC_{50} values were 7.1 or 7.8 μM and > 15 μM for selenosulfate and selenite, respectively, for a 24 h exposure (Table 1). The cell viability curves showed significant differences for all selenium concentrations ≥3 μM with maximal differences up to 50 %. For A375 cells, exposure to selenosulfate and selenite resulted in comparable cell viability curves. Slight differences were observed for the calculated IC_{50} values (6.6 μM for selenosulfate and 4.7 μM for selenite, respectively, Table 1) due to large standard deviations in the mid concentration range. For T24 cells, the cytotoxicity of selenosulfate was lower than that of selenite (IC_{50} values 6.9 or 7.0 and 3.5 μM, Table 1) with maximal differences up to 45 %. It is interesting to note that the cytotoxicity of selenosulfate was rather similar for all three cell lines while the cytotoxicity for selenite increased in the order HepG2 < A375 < T24 cells (Fig. 1).

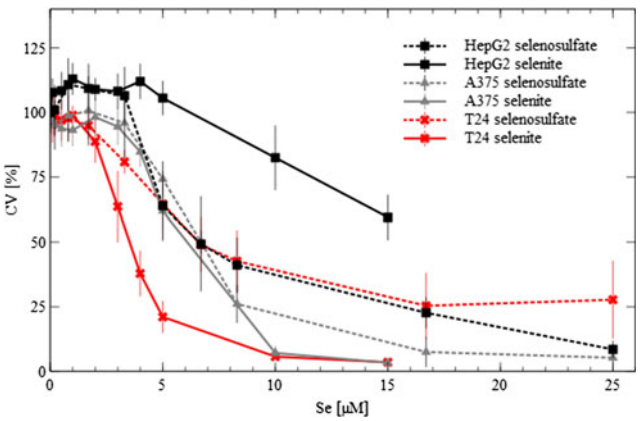

Fig. 1 Cell viabilities (CVs) [%] of HepG2, A375, and T24 cells after 24 h incubation with selenosulfate (0.17–25 μM) and selenite (0.1–15 μM Se), respectively; CVs were determined by MTT assay

For the two cell lines with opposing trends of selenosulfate-selenite toxicity, HepG2 and T24 cells, more detailed investigations were done varying exposure time (24, 48, and 72 h) and in the presence or absence of NEAA. For easier comparison, Fig. 2 (species comparison), Fig. 3 (time comparison), and Fig. 4 (cell comparison) present essentially the same 24 cell viability curves derived from different combinations of these variables.

Looking at the species comparison graph (Fig. 2), it becomes obvious that for HepG2 cells, selenosulfate was always more toxic than selenite. The addition of NEAA had no significant effect on selenosulfate cytotoxicity ($p > 0.19$ for all incubation times, Table SI-1) and only significant influence on selenite cytotoxicity after 48 and 72 h ($p < 0.001$, Table SI-2). For T24 cells, addition of NEAA had an important effect: While selenosulfate curves were almost identical with (IC_{50} 6.9/7.0 μM) or without NEAA (IC_{50} 6.6 μM), selenite became much less toxic in the presence of NEAA (IC_{50} 8.4 μM compared to IC_{50} 3.5 μM in the absence on NEAA). In fact, with the addition of NEAA, selenite was less toxic than selenosulfate for T24 cells just as observed for HepG2 cells. Over time (48 and 72 h versus 24 h), the cell viability curves for both species became more similar, essentially due to a stronger increase in toxicity for selenite and a less pronounced increase in toxicity for

Table 1 IC_{50} values for HepG2, A375, and T24 cells exposed to selenosulfate (0.17–25 μM) or selenite (0.1–15 μM) for 24, 48, and 72 h; 24 h experiments with HepG2 and T24 cells were conducted twice (I and II); growth conditions were changed to test the influence of non-essential amino acids (NEAA), i.e., HepG2 cells were grown without NEAA, T24 with NEAA addition

IC_{50} [μM]		24 h (I)	24 h (II)	48 h	72 h
HepG2 + NEAA	selenosulfate	7.1	7.8	6.0	4.3
	selenite	>15	>15	11.7	4.2
HepG2 - NEAA	selenosulfate		13.8	4.2	3.4
	selenite		>15	4.2	3.0
A375 (no NEAA)	selenosulfate	6.6			
	selenite	4.7			
T24 + NEAA	selenosulfate		6.6	2.8	2.4
	selenite		8.4	2.9	2.4
T24 − NEAA	selenosulfate	6.9	7.0	1.5	1.0
	selenite	3.5	3.5	1.0	1.0

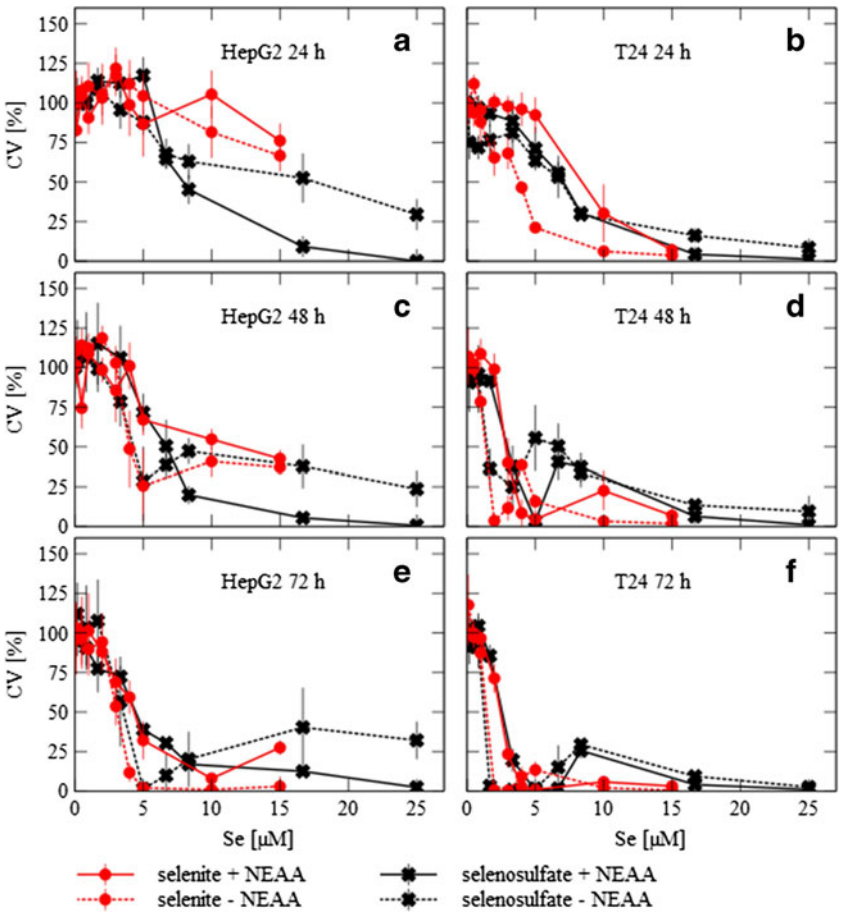

Fig. 2 Comparison of selenosulfate and selenite: Cell viabilities (CVs) after selenosulfate (0.17–25 µM) or selenite incubation (0.1–15 µM) in HepG2 (**a**, **c**, **e**) and T24 cells (**b**, **d**, **f**) after 24 h (**a**, **b**), 48 h (**c**, **d**), and 72 h (**e**, **f**); in addition to treatment with standard growth media, HepG2 cells were cultivated and incubated in growth medium without NEAA, T24 cells were cultivated and incubated in growth medium with NEAA; CVs were determined by MTT assay

selenosulfate (Fig. 2c–f). After 72 h, IC_{50} values were nearly identical with 4.3 µM for selenosulfate and 4.2 µM for selenite for HepG2 cells and 1.0 µM for both selenosulfate and selenite in T24 cells. The absence of NEAA increased cytotoxicity for both species in both cell lines with IC_{50} values being lower than in

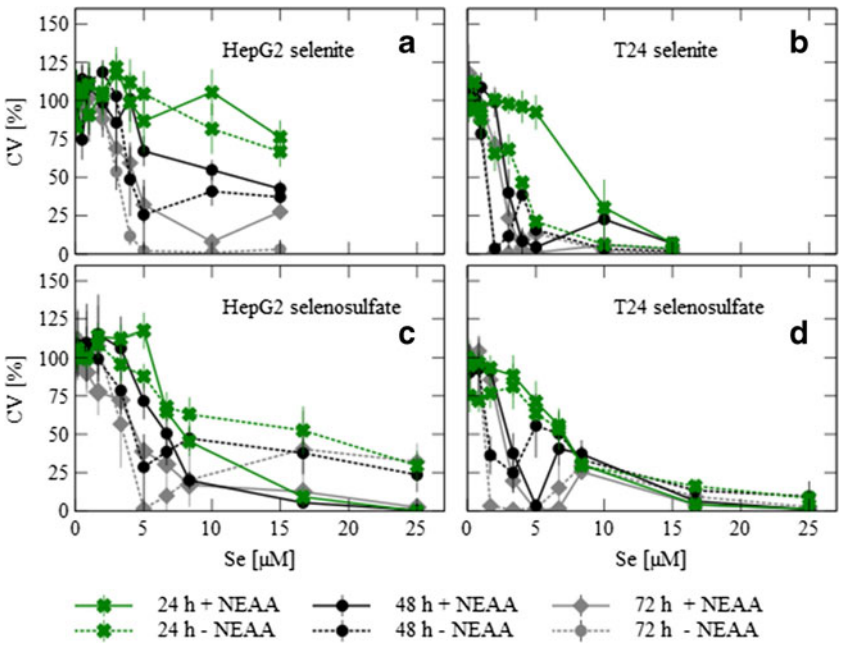

Fig. 3 Comparison of incubation times: Cell viabilities (CVs) after 24 h, 48 h, and 72 h incubation with 0.17–25 µM selenosulfate or 0.1–15 µM selenite in HepG2 (**a**, **c**) and T24 cells (**b**, **d**); in addition to treatment with standard growth media, HepG2 cells were cultivated and incubated in growth medium without NEAA, T24 cells were cultivated and incubated in growth medium with NEAA; CVs were determined by MTT assay

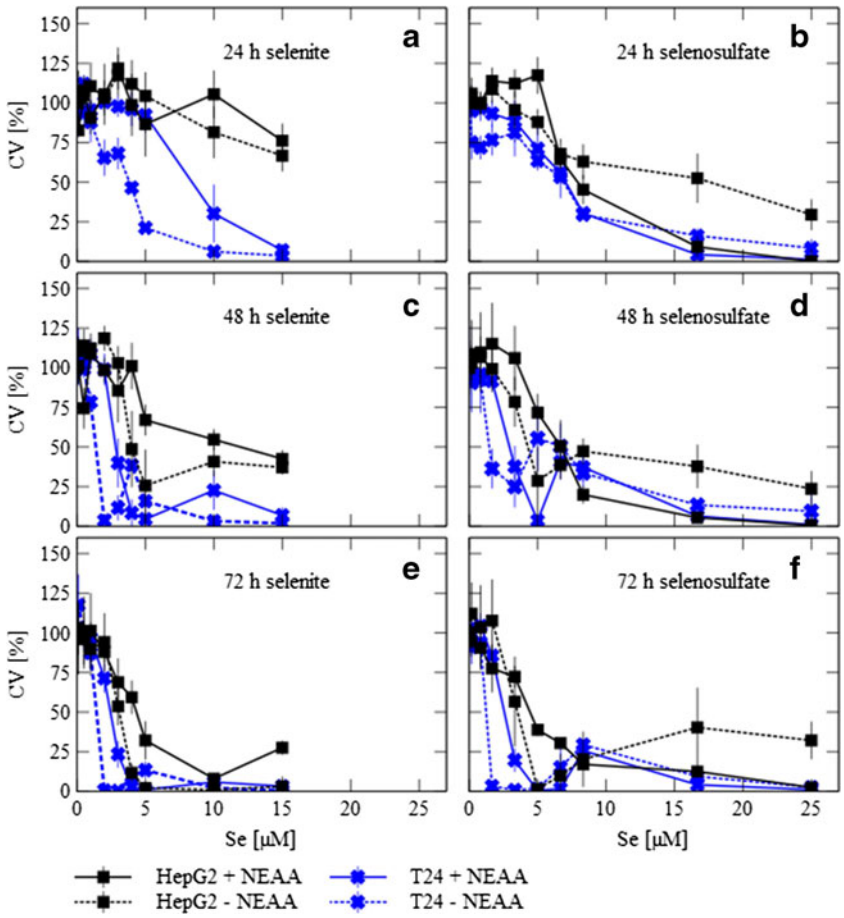

Fig. 4 Comparison of HepG2 and T24 cells: Cell viabilities (CVs) after 24 h (**a**, **b**), 48 h (**c**, **d**), and 72 h (**e**, **f**) incubation with 0.17–25 μM selenosulfate (**b**, **d**, **f**) or 0.1–15 μM selenite (**a**, **c**, **e**); in addition to treatment with standard growth media, HepG2 cells were cultivated and incubated in growth medium without NEAA, T24 cells were cultivated and incubated in growth medium with NEAA; CVs were determined by MTT assay

the presence of NEAA (Table 1) (exception for HepG2 cells after 24 h incubation).

Figure 3 shows nicely the time effect, again. Cell viability curves for each selenium species are presented separately for HepG2 and T24 cells. While for selenosulfate the cell viability curves for 24, 48, and 72 h were quite close together for both cell lines (Fig. 3c, d), there was a significant difference between 24 and 48 h ($p < 0.001$, Table SI-2) as well as between 48 and 72 h ($p < 0.002$) independent of NEAA absence or presence for selenite in HepG2 cells (Fig. 3a). The selenite toxicity in HepG2 cells after 24 h of exposure was remarkably low with cell viabilities of ≥ 67 % even at concentrations of 15 μM. In T24 cells, selenite after 24 h exposure in the presence of NEAA was the curve most prominently distinct from all others (Fig. 3b).

Comparing the general susceptibility of HepG2 versus T24 cells shows that HepG2 cells are more robust for both species and over all exposure times (Fig. 4). It is, however, also obvious that this difference is even more pronounced for selenite than for selenosulfate. Figure 4 also shows again that addition of NEAA has a larger effect on selenite compared to selenosulfate, especially for short-term exposure (24 h) and the more susceptible T24 cells (Fig. 4a).

In contrast to what was observed for HepG2 and T24 cells, no cellular Se retention could be determined for A375 cells after 24 h exposure to 1 μM selenite or 1.7 μM selenosulfate as there was no difference between intracellular selenium concentrations of non-treated A375 control cells (0.73 ng/10^6 cells) or A375 cells exposed to selenosulfate (0.65 ng/10^6 cells) or selenite (0.71 ng/10^6 cells) (Table 2). However, exposure to low Se concentrations (< 5 μM) were associated with cytotoxic effects that were even more pronounced than in HepG2 cells (Fig. 1, Table 1).

For HepG2 and T24 cells, intracellular selenium concentrations were above those of non-treated control cells and there was a significant difference for the two selenium species. For selenite, uptake was always greater in T24 cells than in HepG2 cells. The addition of NEAA enhanced the difference between the two cell lines with 8.5 ng/10^6 cells in T24 cells in the absence of NEAA compared to only 2.7 ng/10^6 cells in HepG2 cells in the presence of NEAA. The order of increasing intracellular concentrations corresponds to the increasing toxicity and decreasing IC_{50} values (Fig. 4a). While differences in intracellular concentrations were small (maximum differences 8.5–2.7 = 5.8 ng/10^6 cells), differences in IC_{50} values were large (> 11.5 μM) (Table 1). For selenosulfate, intracellular concentrations showed a wider range between the

Table 2 Intracellular selenium content [ng Se/10^6 cells/μM of applied Se] in HepG2, A375, and T24 cells; cells were incubated with 1.7 μM selenosulfate or 1 μM selenite for 24 h prior to mechanical lysis and analysis of total selenium content of the cell lysates by ICP-MS. Growth conditions were changed to test the influence of non-essential amino acids (NEAA), i.e., HepG2 cells were grown without NEAA, T24 with NEAA addition

Intracellular Se [ng/10^6 cells/μM of applied Se]	+NEAA	−NEAA
HepG2 selenosulfate	23.2	3.2/2.5*
HepG2 selenite	2.7	4.5/4.0*
HepG2 without selenium addition (control)	1.5	
A375 selenosulfate		0.65
A375 selenite		0.71
A375 without selenium addition (control)		0.73
T24 selenosulfate	1.7/1.5*	12.9
T24 selenite	6.2/2.8*	8.5
T24 without selenium addition (control)		0.5

*Experiments were conducted in duplicate

two cell lines with the lowest concentrations of 1.5 ng/10^6 cells in HepG2 cells, interestingly also in the presence of NEAA. Uptake in the absence of NEAA was surprisingly lower (3.2/2.5 ng/10^6 cells). The wider range of intracellular selenium after selenosulfate exposure (maximum difference 23.2–1.5 = 21.7 ng/10^6 cells) is not reflected in the quite comparable cell viability curves (maximum difference of IC_{50} values 13.8–6.6 = 7.2 μM) (Fig. 4b, Table 1). If we compare the effects in the "routinely" applied media (i.e., with NEAA for HepG2 and without NEAA for T24 cells), intracellular selenite concentrations were lower (2.7 versus 8.5 ng/10^6 cells) in HepG2 than in T24 cells.

Discussion

Based on the results of our study we have to caution that the claim of patent US 2010/0172822 that "sodium selenosulfate had a much stronger cytotoxicity to tumor cells than sodium selenite" (Zhang 2010) cannot be generalized. While selenosulfate was more toxic than selenite in previous cytotoxicity tests with tumor cells of the liver (HepG2), the intestine (Caco-2), and the blood (HL60, T lymph adenoma, Daudi) (Zhang et al. 2008a), we showed that for tumor cells of the skin (A375) and the bladder (T24) this is not the case.

Our study showed that the selenosulfate toxicity was similar between HepG2, A375, and T24 cells (Fig. 1) and largely independent of exposure time or absence and presence of amino acids, despite great variations in intracellular concentrations. This is in contrast to a previous study (Olm et al. 2009) where selenium uptake was found to determine its extent of cytotoxicity in different cell lines. What changed among the three cell lines was the toxicity of selenite, which was lower than that of selenosulfate only for HepG2 cells, but similar to and higher than that of selenosulfate for A375 and T24 cells, respectively.

HepG2 cells were generally the least susceptible cell line among the three cell lines tested, which is in accordance with previous observations from our own studies on cytotoxicity of arsenite and thioarsenates ($AsS_{4-n}O_n^{3-}$) (Hinrichsen et al. 2014). Especially at short exposure times, selenite toxicity to HepG2 cells was remarkably low, both compared to selenosulfate in HepG2 cells but also to selenite toxicity in A375 and T24 cells. Over time, selenite toxicity increased much more than that of selenosulfate and for longer exposure times, toxicities for both species became similar. In this context, it is interesting to note that the cytotoxicities that Zhang et al. (2008a) measured after 72 h for HepG2 cells were more comparable to what we measured after 24 h. In the present study, 5 μM selenosulfate resulted in 117 % CV and 5 μM selenite resulted in 87 % CV. In the study of Zhang et al. (2008a), 6 μM selenosulfate resulted in 80 % CV and 6 μM selenite resulted in 90 % CV after 72 h. The difference between both studies is that Zhang et al. (2008a) used fresh growth medium for Se incubation whereas we already grew HepG2 cells in the medium 24 h before selenium incubation to maintain the routine of fresh medium supply as applied during the cell cultivation. Thus, it seems that the large difference between selenosulfate and selenite toxicity is only a short-term effect, observable under optimum conditions — short exposure (24 h in our case) or fresh medium (Zhang et al. 2008a, b) — for this specific cell line. An obvious explanation might be the very low selenite uptake compared to selenosulfate uptake in HepG2 or to selenite uptake in T24 cells. Amino acids had little short-term effect on either selenite or selenosulfate cytotoxicity in HepG2 cells but decreased cytotoxicity slightly for longer exposure times.

For T24 cells, we observed the opposite effect as for HepG2 cells that selenite was more toxic than selenosulfate. Small increases in intracellular selenite concentrations had a much higher effect on increasing cytotoxicity both in comparison to selenite in HepG2 cells as well as to selenosulfate in T24 cells. Over time, the difference between selenite and selenosulfate decreased. The addition of NEAA actually even reversed the order of toxicity and decreased cellular uptake of selenite. The strong effect that NEAA had on T24 cells, but much less on HepG2 cells, could be explained by an effect on the cellular transport system x_c^-. Cellular uptake of selenite was previously shown to be highly dependent of the cellular transport system x_c^-. Inhibition of the x_c^- system decreases the extracellular cysteine concentration and therefore, less selenite is reduced to HSe^- leading to decreased uptake and cytotoxicity (Olm et al. 2009). One amino acid that was contained in the NEAA mixture we applied and for which an effect had previously been shown (Olm et al. 2009) is L-glutamic acid. In the form of its salt monosodium glutamate it

had been shown to prevent toxic effects of selenite in lung tumor cells by inhibiting cystine transport in the cells (dependent on the x_c^- transport system) in a competitive manner (Gout et al. 1997; Olm et al. 2009). The x_c^- transport system was reported to be more expressed in selenite-sensitive compared to selenite-resistant lung tumor cells (Olm et al. 2009). The higher susceptibility of T24 compared to HepG2 cells and the higher selenite uptake in T24 cells compared to HepG2 cells after 24 h, let us conclude that the x_c^- transport system is more expressed in T24 than in HepG2 cells and that this is the reason for the stronger effect of presence and absence of NEAA in T24 cells.

In contrast to selenite, selenosulfate cytotoxicity seemed to be less affected by changes in the x_c^- transport system. This could be explained by facilitated uptake because of its reduced state. Compared to selenite with an oxidation state of +4, selenosulfate has an oxidation state of −1. Furthermore, selenosulfate is synthesized from glutathione and sulfite and non-reacted excess of any of these two compounds creates a reductive extracellular microenvironment (Olm et al. 2009). Thiols of excess GSH could also facilitate selenium uptake (Bannai 1986) by formation of further reduced selenium forms in addition to the formed selenosulfate (Ganyc and Self 2008), e.g., selenotrisulfide, selenopersulfide, and hydrogen selenide (Bannai 1986). Transport of these reduced compounds could occur through anion channels of the plasma membrane as reported previously (Conrad and Sato 2012).

Another observation from our study where the growth medium seemed to have governed the toxicity of selenium species for the respective cell line is A375. In contrast to the other two cell lines, intracellular concentrations after both selenite and selenosulfate incubation were not distinguishable from untreated control cells, but caused comparable toxicities as in HepG2 and T24 cells which indicated that A375 cells were especially susceptible to selenium. A potential reason could be that in contrast to HepG2 and T24 cells, A375 cells were cultivated in DMEM medium, which contained about twice as much L-cystine compared to MEM medium. We hypothesize that the presence of extracellular cystine changes the GSH/GSSG ratio, resulting in a decreased defense against oxidative stress produced by selenium. Increased toxicity of oxidative stress producing H_2O_2 in the presence of high extracellular cystine levels was already shown for *E. coli* (Smirnova et al. 2005).

Experiments with human tumor cell lines are a basic pre-clinic test in terms of anticancer drug permission. As already described, compositions of cell growth media differ between laboratories leading to limited comparability of cellular uptake and cytotoxicity values of substances. The results of our study showed that the absence or presence of only one medium supplement — NEAA — can completely change the interpretation of the cytotoxicity for a specific substance.

Conclusion

The claim that selenosulfate is more toxic than selenite for tumor cells and this could be an additional reason for its preferred application in anticancer therapy compared to selenite, cannot be generalized. While this has been demonstrated for HepG2 cells, our cell culture study showed that there are cells for which selenite is at least as toxic (A375 cells) or more toxic (T24 cells) than selenosulfate. Our cell culture studies further showed that the choice of growth medium significantly influences the outcome of cytotoxicity data. Uptake of selenite was shown to be downregulated by the presence of NEAA, especially in selenium-sensitive cells, most likely because L-glutamic acid inhibited the x_c^- transport system. The uptake of selenosulfate, on the other hand, was generally facilitated by it being the more reduced species. We conclude that whether selenite or selenosulfate is more toxic to a specific tumor cell line depends largely on the sensitivity of each cell line as well as the reductive state of the extracellular environment. The study shows the importance of considering the effects supplements in cell growth media might have on uptake and cytotoxicity of different compounds for individual cell lines.

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References

Baldew GS, Vandenhamer CJA, Los G, Vermeulen NPE, Degoeij JJM, McVie JG (1989) Selenium-induced protection against cis-diamindichloroplatinum (II) nephrotoxicity in mice and rats. Cancer Res 49:3020–3023

Bannai S (1986) Exchange of cystine and glutamate across plasma membrane of human fibroblasts. J Biol Chem 261:2256–2263

Brinkman M, Buntinx F, Muls E, Zeegers MP (2006) Use of selenium in chemoprevention of bladder cancer. Lancet Oncol 7:766–774

Camargo SMR, Francescato HDC, Lavrador MAS, Bianchi MLP (2001) Oral administration of sodium selenite minimizes cisplatin toxicity on proximal tubules of rats. Biol Trace Elem Res 83:251–262

Chen T, Wong YS (2008) Selenocystine induces apoptosis of A375 human melanoma cells by activating ROS-mediated mitochondrial pathway and p53 phosphorylation. Cell Mol Life Sci 65:2763–2775

Chen T, Wong YS, Zheng W, Bai Y, Huang L (2008) Selenium nanoparticles fabricated in Undaria pinnatifida polysaccharide solutions induce mitochondria-mediated apoptosis in A375 human melanoma cells. Colloids Surf B Biointerfaces 67:26–31

Chen XJ, Duan FD, Zhang HH, Xiong Y, Wang J (2012) Sodium selenite-induced apoptosis mediated by ROS attack in human osteosarcoma U2OS cells. Biol Trace Elem Res 145:1–9

Chu H-A, Crawford-Brown DJ (2006) Inorganic arsenic in drinking water and bladder cancer: a meta-analysis for dose-response assessment. Int J Environ Res Public Health 3:316–22

Conrad M, Sato H (2012) The oxidative stress-inducible cystine/glutamate antiporter, system xc−: cystine supplier and beyond. Amino Acids 42:231–246

Dennert G, Horneber M (2006) Selenium for alleviating the side effects of chemotherapy, radiotherapy and surgery in cancer patients. The Cochrane database of systematic reviews, CD005037

Ganyc D, Self WT (2008) High affinity selenium uptake in a keratinocyte model. Febs Letters 582:299–304

Gout PW, Kang YJ, Buckley DJ, Bruchovsky N, Buckley AR (1997) Increased cystine uptake capability associated with malignant progression of Nb2 lymphoma cells. Leukemia 11:1329–1337

Hinrichsen S, Lohmayer R, Zdrenka R, Dopp E, Planer-Friedrich B (2014) Effect of sulfide on the cytotoxicity of arsenite and arsenate in human hepatocytes (HepG2) and human urothelial cells (UROtsa). Environ Sci Pollut Res 21:10151–10162

Hippler J, Zdrenka R, Reichel RAD, Weber DG, Rozynek P, Johnen G, Dopp E, Hirner AV (2011) Intracellular, time-resolved speciation and quantification of arsenic compounds in human urothelial and hepatoma cells. J Anal At Spectrom 26:2396–2403

Hogberg J, Kristoferson A (1979) Inhibition of amino-acid uptake in isolated rat hepatocytes by selenite. Febs Letters 107:77–80

Johnen G, Rozynek P, von der Gathen Y, Bryk O, Zdrenka R, Johannes C, Weber DG, Igwilo-Okuefuna OB, Raiko I, Hippler J, Bruning T, Dopp E (2013) Cross-contamination of a UROtsa stock with T24 cells - molecular comparison of different cell lines and stocks. Plos One 8:e64139–e64139

Kellen E, Zeegers M, Buntinx F (2006) Selenium is inversely associated with bladder cancer risk: a report from the Belgian case-control study on bladder cancer. Int J Urol 13:1180–1184

Kim EH, Sohn S, Kwon HJ, Kim SU, Kim M-J, Lee S-J, Choi KS (2007) Sodium selenite induces superoxide-mediated mitochondrial damage and subsequent autophagic cell death in malignant glioma cells. Cancer Res 67:6314–6324

Li G-X, Hu H, Jiang C, Schuster T, Lu J (2007) Differential involvement of reactive oxygen species in apoptosis induced by two classes of selenium compounds in human prostate cancer cells. Int J Cancer 120:2034–2043

Li J, Sun K, Ni L, Wang X, Wang D, Zhang J (2012) Sodium selenosulfate at an innocuous dose markedly prevents cisplatin-induced gastrointestinal toxicity. Toxicol Appl Pharmacol 258:376–383

Lo M, Wang Y-Z, Gout PW (2008) The xc− cystine/glutamate antiporter: a potential target for therapy of cancer and other diseases. J Cell Physiol 215:593–602

Markovic SD, Djacic DS, Cvetkovic DM, Obradovic AD, Zizic JB, Ognjanovic BI, Stajn AS (2011) Effects of acute in vivo cisplatin and selenium treatment on hematological and oxidative stress parameters in red blood cells of rats. Biol Trace Elem Res 142:660–670

McKelvey SM, Horgan KA, Murphy RA (2015) Chemical form of selenium differentially influences DNA repair pathways following exposure to lead nitrate. J Trace Elem Med Biol: Organ Soc Miner Trace Elem (GMS) 29

Micke O, Schomburg L, Buentzel J, Kisters K, Muecke R (2009) Selenium in oncology: from chemistry to clinics. Molecules 14:3975–3988

Olm E, Fernandes AP, Hebert C, Rundlof AK, Larsen EH, Danielsson O, Bjornstedt M (2009) Extracellular thiol-assisted selenium uptake dependent on the xc− cystine transporter explains the cancer-specific cytotoxicity of selenite. Proc Natl Acad Sci U S A 106:11400–11405

Park JS, Ryu JY, Jeon HK, Cho YJ, Park YA, Choi JJ, Lee JW, Kim BG, Bae DS (2012a) The effects of selenium on tumor growth in epithelial ovarian carcinoma. J Gynecol Oncol 23:190–196

Park SH, Kim JH, Chi GY, Kim GY, Chang YC, Moon SK, Nam SW, Kim WJ, Yoo YH, Choi YH (2012b) Induction of apoptosis and autophagy by sodium selenite in A549 human lung carcinoma cells through generation of reactive oxygen species. Toxicol Lett 212:252–261

Peng DG, Zhang JS, Liu QL (2007) Effect of sodium selenosulfate on restoring activities of selenium-dependent enzymes and selenium retention compared with sodium selenite in vitro and in vivo. Biol Trace Elem Res 117:77–88

Planer-Friedrich B, London J, McCleskey RB, Nordstrom DK, Wallschlager D (2007) Thioarsenates in geothermal waters of Yellowstone National Park: determination, preservation, and geochemical importance. Environ Sci Technol 41:5245–5251

Shen HM, Yang CF, Ong CN (1999) Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG2 cells. Int J Cancer 81:820–828

Smirnova GV, Muzyka NG, Oktyabrsky ON (2005) Effects of cystine and hydrogen peroxide on glutathione status and expression of antioxidant genes in Escherichia coli. Biochem (Moscow) 70:926–934

Vernie LN, Bont WS, Emmelot P (1974) Inhibition of in-vitro amino-acid incorporation by sodium selenite. Biochemistry 13:337–341

Vinceti M, Wei ET, Malagoli C, Bergomi M, Vivoli G (2001) Adverse health effects of selenium in humans. Rev Environ Health 16:233–51

Vinceti M, Dennert G, Crespi CM, Zwahlen M, Brinkman M, Zeegers MPA, Horneber M, D'Amico R, Del Giovane C (2014) Selenium for preventing cancer. Cochrane Database of Systematic Reviews

Zhang J (2010) Use of sodium selenosulfate for supplementing selenium and enhancing the therapeutic efficacy of chemotherapy agents, and a rapid process for preparing sodium sulfate

Zhang J, Lu H, Wang X (2008a) MS07116 sodium selenosulfate synthesis and demonstration of its in vitro cytotoxic activity against HepG2, caco2, and three kinds of leukemia cells. Biol Trace Elem Res 125:13–21

Zhang JZ, Peng DG, Lu HJ, Liu QL (2008b) Attenuating the toxicity of cisplatin by using selenosulfate with reduced risk of selenium toxicity as compared with selenite. Toxicol Appl Pharmacol 226:251–259

Zou Y, Niu P, Gong Z, Yang J, Yuan J, Wu T, Chen X (2007) Relationship between reactive oxygen species and sodium-selenite-induced DNA damage in HepG2 cells. Frontiers of medicine in China 1