Biol Trace Elem Res (2013) 151:301–306
DOI 10.1007/s12011-012-9563-x

Preparation of a Novel Organoselenium Compound and Its Anticancer Effects on Cervical Cancer Cell Line HeLa

Pei Guo · Peiguang Zhao · Jing Liu · Hong Ma · Jing Bai · Yu Cao · Yanli Liu · Hongxuan He · Chao Qi

Received: 20 October 2012 / Accepted: 26 November 2012 / Published online: 15 December 2012

Abstract This study aims at developing new organoselenium compounds with good anticancer ability and low biotoxicity. Sucrose selenious ester (sucrose-Se) was synthesized by the reaction between sucrose and selenium oxychloride. MTT assay showed that sucrose-Se effectively inhibited the proliferation of cervical cancer cell line HeLa in a dose-dependent manner without cytostatic influence on human normal liver cell line HL-7702. Morphological observation and agarose gel electrophoresis demonstrated that sucrose-Se induced apoptosis to HeLa cells. In addition, sucrose-Se was able to inhibit proliferation of bladder carcinoma cell line 5637, human malignant melanoma cell line A375, and gastric carcinoma cell line MGC-803. Median lethal dose of sucrose-Se and sodium selenite was 290.0 and 13.1 ppm, respectively, in the acute toxicity test on mice. In conclusion, sucrose-Se has potential in cancer chemoprevention due its apoptosis induction capacity and low biotoxicity.

Keywords Sucrose selenious ester · HeLa cells · Apoptosis · Anticancer

P. Guo · P. Zhao · H. Ma · J. Bai · Y. Cao
Key Laboratory of Pesticide and Chemical Biology (Ministry of Education), College of Chemistry, Central China Normal University, Wuhan 430079, People's Republic of China

J. Liu · Y. Cao (✉) · Y. Liu · C. Qi (✉)
Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Science, Central China Normal University, Wuhan 430079, People's Republic of China
e-mail: caoyu@mail.ccnu.edu.cn
e-mail: qichao@mail.ccnu.edu.cn

H. He (✉)
National Research Center for Wildlife Born Diseases, Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People's Republic China
e-mail: hehx@ioz.ac.cn

Introduction

The protective ability of Se against cancer was suggested in 1969 with the discovery of an inverse relationship between cancer occurrence and Se content of local crop in the USA [1]. Since then, many studies have been focusing on the anticancer effects of different selenium compounds. Although the last cancer prevention trial (SELECT) did not support the positive effects of Se on prostate cancer prevention [2], most studies denoted that Se administration improved clinical results and reduced side effects on cancer patients [3–5]. Several mechanisms have been provided to explain the anticancer actions of Se, including the specific inhibition to tumor cell growth by Se metabolites, antioxidative protection by selenoproteins, induction of apoptosis, modulation of carcinogen metabolism, etc. [6–9].

In current researches, the commonly studied and utilized selenocompounds include selenite, selenate, methylselenic acid, and selenoproteins [10]. Inorganic selenite componds, due to their prooxidant character, are able to induce cellular apoptosis by generating oxidative stress. However, selenite is usually more toxic than organic selenium. Therefore, long-term intake of supplements containing selenite may cause negative influences on human health. It is of great significance to develop novel organoselenium compounds and to study their applications in cancer chemoprevention and cancer therapy.

Materials and Methods

Materials Sucrose and sodium selenite were purchased from Sinopharm Chemical Reagent Co., Ltd (China); selenium oxydichloride was purchased from TCI (Japan). RPMI-1640 culture medium and fetal bovine serum (FBS) were obtained from Gibco BRL company (Grand Island,

NE); diphenyltetrazoliumbromide (MTT), acridine orange (AO), ethidium bromide (EB), and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, MO); human cervical carcinoma cell line HeLa, human normal liver cell line HL-7702, bladder cancer cell line 5637, human epidermal malignant melanoma cell line A375, and gastric cancer cell line MGC-803 were purchased from the China Center for Type Culture Collection, Wuhan University, China; and BALB/c male mice were provided by Shanghai Animal Test Center. All other chemicals not mentioned above were analytical grade and purified by the standard methods.

Preparation of Sucrose-Se

Sucrose (0.01 mol) was dispersed in anhydrous pyridine (20 ml) with moderate stirring at 35 °C for 12 h. Selenium oxydichloride was slowly added into pyridine (*v*/*v*=1/50) at 0 °C and then stirred at 25°C for 12 h. The two well-dispersed solutions were mixed and reacted at 35 °C in sealed condition. After 4 h, the crude product was dissolved in DMSO and centrifuged at 16,000 rpm for 10 min. Chloroform was added into the supernatant to precipitate sucrose selenious ester (sucrose-Se). The mixture was centrifuged at 16,000 rpm for 10 min and then sucrose-Se was collected and lyophilized.

The purified sucrose-Se sample (light yellow viscous solid) was characterized by elemental assay, FTIR (Spectrum-2000, PerkinElmer, USA; 25 °C), and ^{13}C NMR (Bruker, German; 600 MHz, 25 °C) experiments.

Cell Culture

Cells were grown in 1-L culture flasks at 37 °C and maintained in RPMI-1640 medium, supplemented with 10 % FBS. All cells are cultured in the CO_2 incubator (Thermo Forma, USA) in the humidified atmosphere of 95 % air and 5 % CO_2. The culture medium was refreshed every 24 h. Cell suspension was obtained by mechanical isolation and then was transferd into cryotubes containing 10 % DMSO and 90 % serum. The cell suspension was cryopreserved using a programmed freezing machine at constant cooling rate of 1 °C/min. Thereafter, the samples were transferred to a liquid nitrogen container and stored.

MTT Assay of HeLa Cells and HL-7702 Cells Treated with Selenocompounds

HeLa cells (1×10^4 cells/well) were cultured in a 96-well plate at 37 °C and maintained in RPMI-1640 medium, supplemented with 10 % FBS for 15 h. Cells were exposed to various concentrations of sodium selenite or sucrose-Se for 72 h. Cells only treated with culture medium was set as the control group. After the supernatant was removed and washed three times with phosphate-buffered solution (PBS), 10 μl MTT (5 mg/ml) and 100 μl medium were added into each well. Cells were incubated for another 4 h and then dissolved in 200 μl DMSO [11]. The absorbance intensity of each sample was measured by using a microplate reader (Bio-Tek, USA) at 570 nm wavelength. Cell inhibition rate (IR) was calculated following formula (1):

$$IR = (A_C - A_E)/A_C \times 100\ \% \quad (1)$$

(A_c: absorbance intensity of the control group; A_E: absorbance intensity of the experimental group).

MTT assay was carried out on human normal liver cell line HL-7702, bladder carcinoma cell line 5637, human malignant melanoma cell line A375, and gastric carcinoma cell line MGC-803 to evaluate the antiproliferative effect of sucrose-Se on normal cell line and other cancer cell lines.

Morphological Observation of HeLa Cells Treated with Selenocompounds

Hoechst 33258 Staining HeLa cells were treated with sodium selenite or sucrose-Se (equivalent Se dose=0.5 ppm). After 72 h incubation, cells were fixed in 4 % paraformaldehyde for 30 min [12] and stained with 10 μg/ml Hoechst 33258 for 10 min at 37 °C. Then, the cells were washed with PBS and their morphologies were observed under a fluorescence microscope (Chongqing Optical Instrument Factory, China).

AO/EB Double Staining After trypsinization, HeLa cells were stained by AO (0.1 mg/ml) for 1 min and then stained by EB (0.1 mg/ml) for 5 min. Cells were washed with PBS and their morphologies were observed under the fluorescence microscope.

Agarose Gel Electrophoresis

After being treated with selenium samples for 72 h, cells were washed with PBS and lysed with ice-cold lysis buffer. Cell samples were maintained at 56 °C in a water bath for 3 h. DNA fragments were extracted by using phenol/chloroform mixture (1/1, *v*/*v*) and then subjected to 1 % agarose gel electrophoresis (Six One Electrophoresis Factory, Beijing, China) at 90 V for 100 min.

Acute Toxicity Test on Mice Treated with Selenocompounds

Healthy BALB/c male mice (4 weeks old, 20–24 g) were pretreated with 8 h fasting and water deprivation prior to the acute toxicity test. Mice in experimental groups were administrated with different concentration of sodium selenite or sucrose-Se. After 24 h, the mortality was recorded and the

median lethal dose (LD_{50}) was measured. All procedures performed on mice have been approved by the Animal Care Committee of Shanghai Animal Test Center (China). All expriments on mice were performed according to the "Principles of Laboratory Animal Care" (NIH publication 85–23, revised 1985) and all mice were cared following the Guide for the Care and Use of Laboratory Animals.

Antitumor Effect of Sucrose-Se on Tumor-Bearing Mice

Healthy BALB/c male mice were subcutaneously injected with 10 ml HeLa cell suspension (1×10^6 cells/ml). After 24 h, mice were divided into five groups with ten mice in each group. The mice in four experimental groups were injected daily with sucrose-Se of various doses (equivalent Se dose=1, 2, 4, and 8 mg/kg) at their enterocoelia. The control group was only injected with physiological saline. Both sucrose-Se injection and physiological saline injection lasted for 7 days. On the 60th day of our experiment, surviving time (day) of each mouse was recorded. In vivo antitumor effect of sucrose-Se on each selenium concentration was represented by using relative surviving time, which was calculated following formula (2):

$$\text{Relative increased surviving time} = \frac{T_E - T_C}{T_C} \quad (2)$$

(T_E: average surviving days of the experimental group; T_C: average surviving days of the control group).

Statistical Analysis

Data were expressed as means±standard deviations (SD) of multi-replicated determinations. Statistics were performed with the SPSS statistics program (SPSS 16.0 for windows). Results were analyzed by one-way analysis of variance with the Student–Newman–Keuls multiple comparisons or *t* test when comparing the differences between the means of two groups at the same time point. Differences were considered to be statistically significant if $P<0.05$.

Results

Se Content and Molecular Structure of Sucrose-Se

Se content of sucrose-Se was 13.0 % as measured by elemental analysis. ^{13}C NMR data of sucrose: $\delta_{C\text{-}6}$=60.9 ppm and $\delta_{C\text{-}5}$=73.3 ppm. ^{13}C NMR data of sucrose-Se: $\delta_{C\text{-}6}$= 64.2 ppm and $\delta_{C\text{-}5}$=72.7 ppm. Selenic esterification was demonstrated to occur on C-6 OH because the esterification of hydroxyl group caused a characteristic downfield shift of α-C and an upfield chemical shift of β-C [13]. FTIR spectrum of sucrose-Se: two new absorption peaks at 924 and 681 cm^{-1}, were, respectively caused by the stretching vibration of Se=O and stretching vibration of Se–OH [14]. Molecular structures of sucrose and sucrose-Se are shown in Fig. 1.

Antiproliferative Effects of Sucrose-Se on Different Cell Lines

Figure 2 showed inhibition rate of HeLa cells and human normal liver HL-7702 cells treated with sodium selenite or sucrose-Se for 72 h. At low Se concentration of 1 ppm, sucrose-Se was less effective to inhibit the proliferation of HeLa cells compared with sodium selenite. But the sucrose-Se-induced inhibition was very similar to that induced by sodium selenite with the tested Se concentration from 2 to 5 ppm (Fig. 2a). Very interestingly, inhibition rate of normal HL-7702 cells treated with sucrose was −1.03 and 4.69 % corresponding to the Se concentration of 0.15 and 1.2 ppm, respectively. But for HL-7702 cells treated with sodium selenite, inhibition rate was as high as 43.64 and 67.27 % corresponding to the Se concentration of 0.6 and 1.2 ppm, respectively (Fig. 2b). Sucrose-Se had no obvious cytostatic effects on 5637 cells, A375 cells, and MGC-803 cells at low concentration (0.04–0.2 ppm Se), but the inhibition rate of these three carcinoma cells significantly increased when Se concentration reached to 1 ppm and showed an increasing trend with the incremental Se concentration (Fig. 2c).

Morphological Observation of Apoptosis Induced by Selenocompounds

Hoechst 33258 is a fluorescent DNA-binding dye used to define nuclear chromatin morphology, which is an index of cellular apoptosis. Sodium selenite and sucrose-Se induced cellular apoptosis, which was characterized by condensed or fragmented nucleus (Fig. 3b, c). In the sucrose-

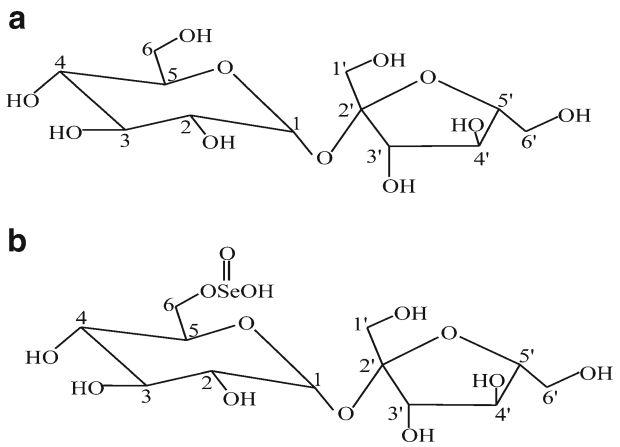

Fig. 1 Molecular structure of sucrose (**a**) and sucrose-Se (**b**)

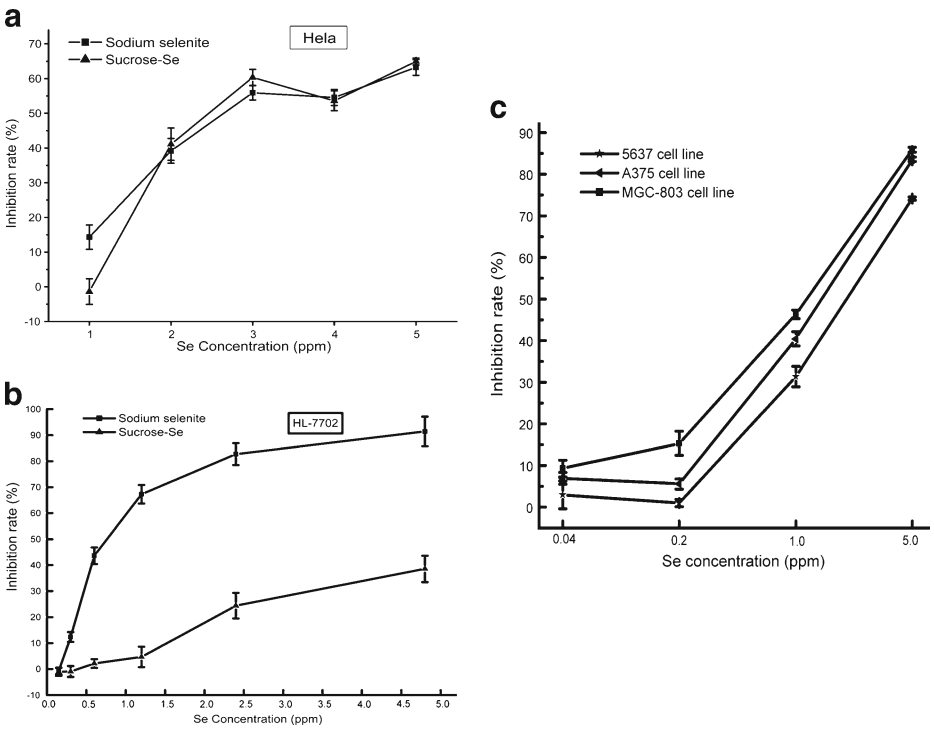

Fig. 2 Inhibition rate of cell lines treated with sodium selenite and sucrose-Se of different concentrations for 72 h. Values were expressed as means±SD of five independent experiments. (**a** inhibition rate of HeLa cells; **b** inhibition rate of HL-7702 cells, $P<0.05$; **c** inhibition rate of bladder carcinoma cell line 5637, human malignant melanoma cell line A375, and gastric carcinoma cell line MGC-803 treated with sucrose-Se)

Se group, more obvious apoptotic bodies were easily observed due to their typical half moon shape. AO/EB double staining is used for parallel detection of cellular apoptosis and necrosis. AO permeates the integrated cell membrane and stains the nucleus to green color. EB can only permeate deteriorated cytoplasmatic membrane and stain the nucleus to red color. Therefore, normal cells are characterized by homogeneous green nucleus, while apoptotic cells can be distinguished by their contracted chromatin and light orange flurescence. Necrotic cells will exhibit unhomogenous orange–red fluorescence. Massive organge–red nucleus and some swollen cells were observed in HeLa cells treated with sodium selenite, indicating the induction of cell necrosis by sodium selenite (Fig. 4b). Cells treated with sucrose-Se showed more obvious apoptotic characters including nuclear condensation and light orange flurescence (Fig. 4c).

Analysis of DNA Fragmentation

The result of agarose gel electrophoresis is shown in Fig. 5. No chromosomal DNA fragments were observed in the control group. But HeLa cells treated with sodium selenite or sucrose-Se group exhibited chromosomal DNA fragments, tailing phenomenon, and formation of DNA ladder.

Biotoxicity of Sucrose-Se and Its Antitumor Effect In Vivo

For normal BALB/c mice treated with sodium selenite or sucrose-Se of different concentrations for 24 h, the LD_{50} of sodium selenite and sucrose-Se was 13.1±2.8 and 290.0±21.2 mg/kg, respectively ($P<0.01$). The relative increased surviving time was 0.75, 1.06, 1.67, and 0.50 for tumor-bearing mice treated with sucrose-Se at equivalent Se dose of 1, 2, 4, and 8 mg/kg, respectively ($P<0.05$).

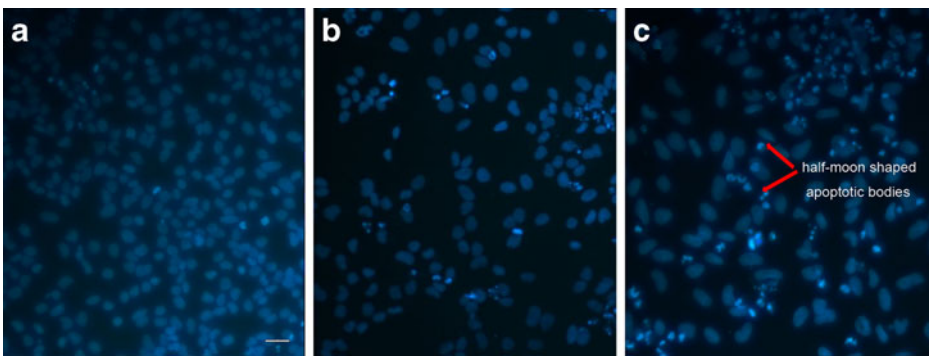

Fig. 3 Morphological observation of HeLa cells treated with sodium selenite and sucrose-Se at Se concentration of 0.5 ppm for 72 h by using the Hoechst 33258 staining method. (**a** control group; **b** sodium selenite group; **c** sucrose-Se group)

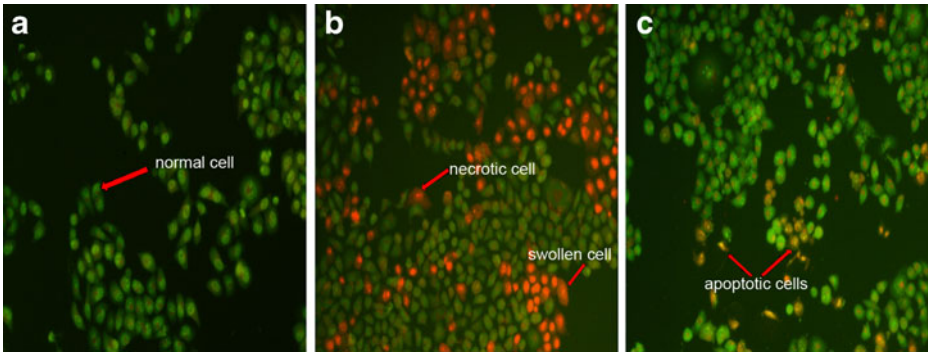

Fig. 4 Morphological observation of HeLa cells treated with sodium selenite and sucrose-Se at Se concentration of 0.5 ppm for 72 h using the AO/EB double-staining method. (**a** control group; **b** sodium selenite group; **c** sucrose-Se group)

Discussion

From the result of MTT assay, both sucrose-Se and sodium selenite exhibited similar antiproliferative effects on HeLa cells with the tested Se concentration from 2 to 5 ppm, showing an obvious dose-dependent manner. More interestingly, sucrose-Se had no obvious antiproliferative effects on normal HL-7702 cells with the tested concentration from 0.15 to 1.2 ppm Se. But in contrast, sodium selenite induced significant inhibition to HL-7702 cells at very low concentration. This is because cellular responses to selenocompounds are both form-dependent and dose-dependent [15]. Considering the cytostatic influences on both cancer cells and normal cells, sucrose-Se is more applicable than sodium selenite to be used in cancer chemoprevention or therapy. In addition, sucrose-Se induced inhibition to bladder carcinoma cell line 5637, human malignant melanoma cell line A375, and gastric carcinoma cell line MGC-803, further demonstrating the broad-spectrum cytostatic effects of sucrose-Se.

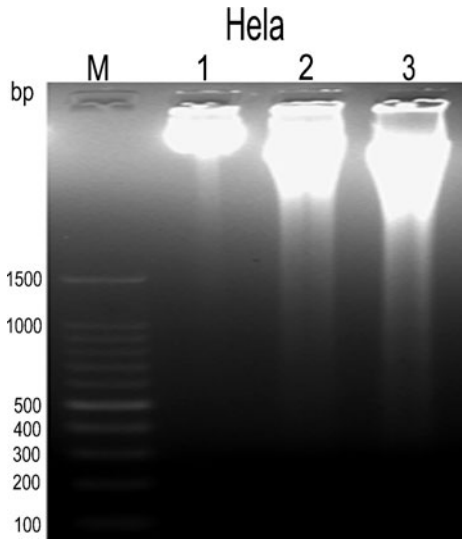

Fig. 5 Gel electrophoretogram of HeLa cells treated with sodium selenite and sucrose-Se with concentration of 0.5 ppm Se for 72 h. (*M* 100 bp DNA maker; *1* control group; *2* sodium selenite group; *3* sucrose-Se group)

Based on the result of morphological observation, sucrose-Se was more efficient to induce apoptosis to HeLa cells, whereas sodium selenite induced more cell necrosis. Cells treated with sucrose-Se exhibited fragmented and condensed chromatin, demonstrating the induction of apoptosis by sucrose-Se. But distinctively, cells treated with sodium selenite exhibited large number of orange nucleus, which are characteristics of necrotic cells at later period. Our finding is also supported by previous report that sodium selenite tends to induce cell necrosis rather than apoptosis [16]. The apoptosis induction ability instead of necrotic induction is considered to be the key factor of a potential anticancer drug [17]. Therefore, the apoptosis induction capacity of sucrose-Se supported its potentials as anticancer drugs. In parallel with the morphological observation, the biochemical features of apoptosis were detected by DNA fragmentation. Cells treated with sucrose-Se (0.5 ppm Se) for 72 h exhibited obvious DNA fragments and tailing phenomenon, indicating that sucrose-Se caused DNA damage to HeLa cells. A mainstream explanation for selenium-induced DNA damage is that selenite and Se-cysteine, precursors of the main selenium metabolite H_2Se, induce DNA single-strand breaks (genotoxicity) [18–21].

LD_{50} measured by acute toxicity test indicated that sucrose-Se possessed much lower biotoxicity than sodium selenite. This novel sucrose-Se has promising potentials in chemopreventive application to substitute the generally used selenite, which may expose an organism to a toxic level.

Sucrose-Se effectively inhibited tumor growth in vivo and extended the surviving time of tumor-bearing mice, compared with the control group. Equivalent Se dose of 4 mg/kg might be the optimum dose of sucrose-Se to inhibit tumor proliferation in mice body. Se dose higher than 4 mg/kg may destroy some normal physiological functions due to the potential toxicity of selenium. This conclusion can be supported by an early study, which indicated that selenium functions as an essential trace nutrient at level of about 0.1 to 0.2 mg/kg in the tested animals, but it became toxic when the level exceeded 5 mg/kg [22].

Summarily, a novel organoselenium compound was synthesized and its chemical structure was identified. Sucrose-Se inhibited proliferation of HeLa cells, bladder carcinoma

cell line 5637, human malignant melanoma cell line A375, and gastric carcinoma cell line MGC-803. However, sucrose-Se had no cytostatic effects on human normal liver cell line HL-7702 at the tested concentration from 0.15 to 1.2 ppm Se. Besides, sucrose-Se induced apoptosis to HeLa cells and possessed much lower toxicity than sodium selenite. Most importantly, sucrose-Se inhibited tumor growth in vivo and extended surviving time of tumor-bearing mice. Taken together, this novel sucrose-Se has promising potentials as anticancer drugs due to its cytostatic effects, apoptosis induction capacity, and low biotoxicity.

Acknowledgments This work was supported by the Scientific Research Foundation for the Returned Overseas Chinese Scholars (State Education Ministry, 2010-1174), Self-Determined Research Funds of CCNU from the Colleges' Basic Research and Operation of MOE (CCNU11A02013), and the project of Hubei Key Laboratory of Genetic Regulation and Integrative Biology (201002).

References

1. Schrauzer GN (2000) Anticarcinogenic effects of selenium. Cell Mol Life Sci 57:1864–1873
2. Lippman SM, Klein EA, PJ Goodman MS et al (2009) Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). JAMA 301:39–51
3. Clark LC, Combs GF Jr, Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham GF, Gross EG, Krongrad A, Lesher JL Jr, Park HK, Sanders BB Jr, Smith CL, Taylor JR (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group JAMA 276:1957–63
4. Clark LC, Marshall JR (2001) Randomized, controlled chemoprevention trials in populations at very high risk for prostate cancer: elevated prostate-specific antigen and high-grade prostatic intraepithelial neoplasia. Urology 57:185–187
5. Duffield-Lillico AJ, Slate EH, Reid ME et al (2003) J Natl Cancer Inst (Cary) 95:1477–1481
6. Ip C, Dong Y, Ganther HE (2002) New concepts in selenium chemoprevention. Cancer Metastasis Rev 21:281–289
7. Schrauzer GN (2009) Selenium and selenium-antagonistic elements in nutritional cancer prevention. Crit Rev Biotechnol 29:10–17
8. Lu JX, Jiang C (2005) Selenium and cancer chemoprevention: hypotheses integrating the actions of selenoproteins and selenium metabolites in epithelial and non-epithelial target cells. Antioxidants & Redox Signaling 7:1715–1727
9. Zeng HW, Briske-Anderson M, Idso JP, Hunt CD (2006) The selenium metabolite methylselenol inhibits the migration and invasion potential of HT1080 tumor cells. J Nutr 136:1528–1532
10. Schrauzer GN (2001) Nutritional selenium supplements: product types, quality, and safety. J Am Coll Nutr 20:1–4
11. Raff MC (1992) Social controls on cell survival and cell death. Nature 356:397–400
12. Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370
13. Pastoriza MA, Bertorello HE (1986) 13C NMR study of the structure of the hydrogel polyester of sucrose methacrylate (Sucro-Gel H-70). Polym Bull 15:241–46
14. Socrates G (2001) Infrared and Raman characteristic group frequencies tables and charts. Wiley, New York
15. Brozmanova J, Manikova D, Vlckova V, Chovanec M (2010) Selenium: a double-edged sword for defense and offence in cancer. Arch Toxicol 84:919–938
16. Freitas M, Alves V, Sarmento-Ribeiro AB, Mota-Pinto A (2011) Combined effect of sodium selenite and docetaxel on PC3 metastatic prostate cancer cell line. Biochem Biophys Res Commun 408:713–719
17. Zeng H, Combs GF Jr (2008) Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. J Nutr Biochem 19:1–7
18. Zhang S, Luo Y, Zeng H et al (2011) Encapsulation of selenium in chitosan nanoparticles improves selenium availability and protects cells from selenium-induced DNA damage response. J Nutr Biochem 22:1137–1142
19. Garberg P, Stahl A, Warholm M, Hogberg J (1988) Studies of the role of DNA fragmentation in selenium toxicity. Biochem Pharmacol 37:3401–06
20. Snyder RD (1987) Effects of sodium selenite on DNA and carcinogen-induced DNA repair in human diploid fibroblasts. Cancer Lett 34:73–81
21. Wilson AC, Thompson HJ, Schedin PJ et al (1992) Effect of methylated forms of selenium on cell viability and the induction of DNA strand breakage. Biochem Pharmacol 43:1137–41
22. Jacobs M, Forst C (1981) Toxicological effects of sodium selenite in Sprague–Dawley rats. J Toxicol Environ Heal 8:575–85