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Synergism between thioredoxin reductase inhibitor ethaselen and sodium selenite in inhibiting proliferation and inducing death of human non-small cell lung cancer cells

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ABSTRACT

New effective treatment for human non-small cell lung cancer (NSCLC) is needed. The thioredoxin (Trx) system composes of thioredoxin reductase (TrxR), Trx and NADPH. In this study, we combined an organic selenium compound–TrxR inhibitor ethaselen (BBSKE) with low dosage sodium selenite to inhibit proliferation and induce death of NSCLC cells, and identified underlying mechanisms. Synergistic anti-proliferation effect of BBSKE and selenite was found in human NSCLC cell lines, A549, NCI-H1299 and NCI-1266. A significant increase of apoptosis, necrosis and autophagy were observed in the group of BBSKE plus selenite in A549 cells. The autophagy induced by BBSKE and selenite inhibited apoptosis and necrosis. In addition, BBSKE plus selenite induced G2/M arrest, which was verified by the alteration of gene and protein expression of cell cycle regulatory complexes. The intracellular enzyme activity of TrxR was remarkably decreased by cotreatment of BBSKE and selenite. Besides, the mRNA and protein level of TrxR1 and Trx1 were significantly inhibited by cotreatment of BBSKE and selenite. HEK 293 cells over-expressing TrxR1 were more sensitive to BBSKE plus selenite. The nuclear translocation of Trx1 and Ref-1, as well as expression of Ref-1 and AP-1 were inhibited by combination treatment. In short, BBSKE synergizes selenite in inhibiting proliferation and inducing death of NSCLC cells; BBSKE combined with selenite may be a treatment strategy for NSCLC.

1. Introduction

The morbidity and mortality of lung cancer rank first among all human malignancies worldwide. About 80% of lung cancers fall into the category of non-small cell lung cancer (NSCLC), meanwhile approximately 70% NSCLC patients had already progressed to advanced stage when being diagnosed [1]. The first-line treatment of NSCLC is mainly focused on cytotoxic chemotherapy with severe toxic and adverse events; targeted agents, such as gefitinib and erlotinib against epidermal growth factor receptor (EGFR), are commonly used for EGFR-mutated NSCLC, while NSCLC cells are very likely to acquire resistance to targeted therapy [2]. Therefore, new effective treatment approaches to NSCLC are urgently needed.

The thioredoxin (Trx) system, composed of Trx, thioredoxin reductase (TrxR) and NADPH, plays an important role in regulating cellular redox homeostasis [3]. TrxR receives hydrogen from NADPH and reduces a broad spectrum of substrates, including Trx-S_2. In addition, Trx regulates activities of some transcription factors by inducing their translocation from cytosol to nucleus, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [4]. It is reported that TrxR1 located in cytoplasm is over-expressed in many malignant tumor tissues compared with surrounding normal tissues, including lung cancer, breast cancer, prostate and colorectal carcinoma etc. [5,6]. Trx system promotes tumor development and progression by facilitating tumor cells growth and proliferation,

Abbreviations: Trx, thioredoxin; TrxR, thioredoxin reductase; NSCLC, non-small cell lung cancer; BBSKE, ethaselen; Ref-1, redox factor 1; AP-1, activator protein-1; GSH, glutathione; ROS, reactive oxygen species; NF-κB, nuclear factor-κB; GR, glutathione reductase; GPx, glutathione peroxidase; CDK, cyclin-dependent kinase; PI, propidium iodide; CQ, chloroquine; 3-MA, 3-methyladenine; LC3, light chain 3.

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evading antigrowth signals and apoptosis, sustaining angiogenesis and so on [7,8]. Hence, TrxR1 has been regarded as a promising cancer diagnostic marker and therapeutic target with inhibitors under research and development [7].

Ethaselen (BBSKE) is a selective TrxR inhibitor containing selenium, which has been shown to directly inhibit TrxR activity, induce reactive oxygen species (ROS) and promote cancer cell apoptosis [9,10]. The cancer prevention and therapeutic effect of selenium-containing compounds remain controversial, especially in randomized control trials [11], which may be explained by different serum selenium concentrations of the study participants [12]. Recent studies provide evidences that selenium supplementation improves compliance and decreases treatment discontinuations in patients with cancer, particularly in selenium-deficient ones [12]. In cancer chemoprevention and therapy studies, sodium selenite has been demonstrated to be able to produce ROS during metabolism [13,14], and selenium-containing compounds could improve efficacy of anticancer drugs or radiation [15]. However, the anticancer effect of organic combined with inorganic selenium compounds, incorporating with a selenoprotein, has rarely been explored. Xu et al. showed that sodium selenite at non-toxic concentration could significantly sensitize A549 lung cancer cells towards BBSKE [16]. Cotreatment significantly reduced free thiols and glutathione (GSH), leading to improved intracellular ROS level and cytotoxicity compared with either single treatment. However, the molecular mechanism of cotreatment with low dosage sodium selenite and BBSKE deserves further investigation in deed. In the present study, we aim to confirm the synergistic effect of antiproliferation and death induction of BBSKE and sodium selenite in human NSCLC cell lines. Besides, we explore the underlying mechanisms to expand the understanding of cotreatment of sodium selenite and a selenium-containing targeted compound, providing evidences for potential new effective treatment of NSCLC.

2. Methods and materials

2.1. Chemicals and drugs

Ethaselen (BBSKE) was designed and synthesized in State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, China [17]. Sodium selenite (1 mg/ml) was purchased from Macgene (Beijing, China). BBSKE was dissolved in DMSO with 5 mM as stock solution. Autophagy inhibitors chloroquine (CQ) was obtained from Sigma-Aldrich (St. Louis, MO, USA), 3-methyladenine (3-MA) was obtained from Harveybio (Beijing, China).

2.2. Cell culture, cell viability assay and Chou-Talalay median-effect analyses

NSCLC cell lines A549 and NCI-H1299, normal lung fibroblast cell line MRC-5 and mammary epithelial cell line MCF-10 A were obtained from Cell Resource Center at Peking Union Medical College (Beijing, China). NCI-H1666 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HEK 293 cells stably overexpressing TrxR1 were produced in our lab [18] as previously described [19]. HEK 293-TrxR1 and HEK 293-EGFP cells (control) were grown in DMEM-F12 (Macgene, Beijing, China) and NSCLC cells were cultured in DMEM (Macgene, Beijing, China), both were supplemented with 10% fetal bovine serum (Biological Industries, Israel). MCF-10A cells were cultured in phenol red-free DMEM-F12 (Macgene, Beijing, China) as previously described [20]. MRC-5 cells were grown in MEM/EBSS (Hyclone, Logan, Utah) supplemented with 1% nonessential amino acids (Macgene, Beijing, China) and 10% FBS. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The effects of sodium selenite, BBSKE and cotreatment against NSCLC cell lines, HEK 293 cells, MCF-10A and MRC-5 cells were measured by sulforhodamine B (SRB) assay in parallel as described [16]. Chou-Talalay median-effect analysis was performed with pooled results of three independent experiments by CompuSyn software to investigate the interaction effect of selenite and BBSKE in NSCLC cell lines [21]. Combination index (CI) was calculated by the following equation: $CI = D_1/(Dx)_1 + D_2/(Dx)_2$, D_1 and D_2 were the doses necessary to achieve a particular effect in combination, $(Dx)_1$ and $(Dx)_2$ referred to the doses of the same drug with D_1 and D_2 respectively to produce the identical effect alone. Values of CI reflect different ways of drugs interaction: $CI < 0.9$, $0.9 < CI < 1.1$ and $CI > 1.1$ indicate synergism, additive effect and antagonism respectively.

2.3. Apoptosis assay with annexin V-PI

Apoptosis assay was conducted as previously descripted [22]. Briefly, A549 cells (5.4×10^5/dish) were seeded in 60 mm dishes and cultured for 24 h before exposed to 0.5 μM sodium selenite, 3 μM BBSKE or a combination treatment for 12 h or 24 h. After incubation, cells were harvested with 500 μl trypsin-EDTA 0.25% (Macgene, Beijing, China), combined with suspended cells and centrifuged at 1000 *g* for 2 min. Cells were re-suspended with 185 μl binding buffer (2.5 mM $Cacl_2$, 140 mM NaCl and 10 mM HEPES/HCl pH 7.4) before transferred to 5 ml culture tubes (BD Falcon, Franklin Lakes, NJ, USA). After adding 5 μl Annexin V-FITC (Baosai, Beijing, China; $\lambda_{exc} = 488$ nm and $\lambda_{em} = 525$ nm), cells were incubated in the dark for 10 min at room temperature, followed by addition of 20 μl 500 μg/ml propidium iodide (PI) solution (Sigma-Aldrich, St. Louis, MO, USA; $\lambda_{exc} = 535$ nm and $\lambda_{em} = 615$ nm) and immediate detection by flow cytometry (BD FACS Calibur, Franklin Lakes, NJ, USA).

2.4. Acidic vesicular organelles detection

Acidic vesicular organelles was detected by acridine orange staining. A549 cells were incubated with 1 μg/ml acridine orange (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 15 min after compounds treatment for 24 h, then analyzed by fluorescent microscopy (Olympus, Tokyo, Japan).

2.5. Necrosis detection

Necrosis analysis was conducted as described [23]. Briefly, A549 cells were incubated with Hoechst 33342 (Solarbio, Beijing, China) with a final concentration of 10 μg/ml at 37 °C for 10 min before stained with PI (10 μg/ml) for 20 min at 4 °C. Images were collected with fluorescent microscopy.

2.6. Enzymes activity assay

The whole cell protein was extracted with RIPA buffer (Applygen, Beijing, China) [9] and the protein concentration was determined by BCA method. The enzyme activities were measured by microplate reader (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA) and the absorbances were determined every 15 s for consecutive 30 times (7.5 min in total). For the measurement of each enzyme activity, background reaction was determined by replacing NADPH with sodium phosphate buffer in the control experiment. The enzyme activities of samples were calculated by subtracting the background value. Experiments were run in triplicate.

Determination of TrxR [24] and glutathione reductase (GR)