Free Radical Biology & Medicine 52 (2012) 898–908

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Ethaselen: a potent mammalian thioredoxin reductase 1 inhibitor and novel organoselenium anticancer agent

Lihui Wang [a,b,c], Zhiyu Yang [a,b], Jianing Fu [a,b], Hanwei Yin [d], Kun Xiong [d], Qiang Tan [b,e], Hongwei Jin [a], Jing Li [a,b], Tianyu Wang [d], Wanchen Tang [a,b], Jin Yin [a,b], Gaoxiong Cai [a,b], Mi Liu [a,b], Sebastian Kehr [c], Katja Becker [c], Huihui Zeng [a,b,*]

[a] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100191, China
[b] School of Pharmaceutical Sciences, Peking University, Beijing 100191, China
[c] Interdisciplinary Research Center, Justus Liebig University, Giessen D-35392, Germany
[d] College of Life Sciences, Peking University, Beijing 100075, China
[e] Peking University First Hospital, Beijing 100034, China

ARTICLE INFO

Article history:
Received 7 July 2011
Revised 14 November 2011
Accepted 29 November 2011
Available online 21 December 2011

Keywords:
Mammalian thioredoxin reductase 1
Thioredoxin
Ethaselen
Enzyme inhibitor
Selenium compound
Anticancer agent
Free radicals

ABSTRACT

Mammalian thioredoxin reductase 1 (TrxR1) is considered to be an important anticancer drug target and to be involved in both carcinogenesis and cancer progression. Here, we report that ethaselen, a novel organoselenium compound with anticancer activity, specifically binds to the unique selenocysteine–cysteine redox pair in the C-terminal active site of mammalian TrxR1. Ethaselen was found to be a potent inhibitor rather than an efficient substrate of mammalian TrxR1. It effectively inhibits wild-type mammalian TrxR1 at submicromolar concentrations with an initial mixed-type inhibition pattern. By using recombinant human TrxR1 variants and human glutathione reductase, we prove that ethaselen specifically targets the C-terminal but not the N-terminal active site of mammalian TrxR1. In A549 human lung cancer cells, ethaselen significantly suppresses cell viability in parallel with direct inhibition of TrxR1 activity. It does not, however, alter either the disulfide-reduction capability of thioredoxin or the activity of glutathione reductase. As a downstream effect of TrxR1 inactivation, ethaselen causes a dose-dependent thioredoxin oxidation and enhances the levels of cellular reactive oxygen species in A549 cells. Thus, we propose ethaselen as the first selenium-containing inhibitor of mammalian TrxR1 and provide evidence that selenium compounds can act as anticancer agents based on mammalian TrxR1 inhibition.

The thioredoxin system, composed of thioredoxin reductase (TrxR), thioredoxin (Trx), and NADPH, regulates various cellular processes, including antioxidant defense, redox homeostasis, cell proliferation, and apoptosis [1,2]. Thioredoxin reductase belongs to the family of pyridine nucleotide disulfide oxidoreductases, together with enzymes such as lipoamide dehydrogenase and glutathione reductase [3]. In mammals three TrxRs have been identified: (i) cytosolic TrxR1, (ii) mitochondrial TrxR2, and (iii) testis-specific TrxR3, which contains an additional N-terminal glutaredoxin domain [4]. All three mammalian TrxRs are selenocysteine-containing enzymes that catalyze the selenium-dependent reduction of the oxidized active-site disulfide (Cys32/Cys35) in thioredoxin [4]. Interestingly, increasing evidence indicates that TrxR1 plays important roles in the development and progression of cancer [1,5]. Many malignant cells overexpress TrxR1 and show markedly enhanced TrxR1 activity, possibly correlating with tumor aggressiveness, apoptosis inhibition, and increased resistance to chemotherapeutic treatment [1,4,6]. It was reported that reduction of TrxR1 levels in cancer cells reverses many characteristics of malignancy and inhibits cancer cell growth and DNA replication [7,8]. Further studies substantiate the notion that mammalian TrxR1 is a promising drug target for cancer therapy [9–11].

Mammalian TrxRs possess an N-terminal dithiol/disulfide redox motif with striking homology to human glutathione reductase (GR) [12]. However, mammalian TrxRs additionally possess a unique and conserved cysteine–selenocysteine (Cys–Sec) redox pair at the C-terminal active site [13,14]. It is believed that mammalian TrxR1 is able to reduce a variety of diverse substrates because of the inherent high reactivity and redox properties of the selenocysteine residue [13,14]. During catalysis electrons are transferred from NADPH via enzyme-bound FAD to the N-terminal active site, from where they are delivered to the oxidized C-terminal arm, reducing the

Abbreviations: ethaselen, 1,2-[bis(1,2-benzisoselenazolone 3(2*H*)-ketone)] ethane; GR, glutathione reductase; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione disulfide; ROS, reactive oxygen species; Sec, selenocysteine; Trx, thioredoxin; TrxR, thioredoxin reductase.

* Corresponding author at: School of Pharmaceutical Sciences, Peking University, Beijing 100191, China. Fax: +86 10 62015584.
E-mail address: zenghh@bjmu.edu.cn (H. Zeng).

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.11.034

selenylsulfide (S–Se, formed by Cys497/Sec498) to a selenolthiol (–SH/–SeH). The current idea is that the reduced Sec498 residue attacks the active-site disulfide of Trx to form an intermolecular selenenylsulfide bond (Se–S, formed by TrxR Sec498 and Trx Cys32). Then the intermolecular selenylsulfide bond is reduced by Cys497 of TrxR via a thiol/selenenylsulfide exchange. As a result, reduced Trx is released and the oxidized C-terminal selenenylsulfide of TrxR is regenerated for the next cycle of catalysis [4,15]. As mentioned above, the C-terminal Sec is highly reactive and solvent exposed, which is thought to be the reason for the broad substrate specificity of mammalian TrxRs, at least in vitro [4,16]. Moreover, the inhibitory effects of many active compounds and drugs targeting mammalian TrxR1 are mainly based on their interactions with the Sec residue; prominent examples include gold- and platinum-based compounds, arsenic trioxide, motexafin gadolinium, antitumor quinols, curcumin, and natural flavonoids. In fact, inactivation of TrxR1 is considered to be involved in the described anticancer effects of these compounds [17–22].

Selenium compounds that are effective as anticancer agents are increasingly gaining interest for the development of new powerful cancer treatment strategies [23]. Ethaselen (1,2-[bis(1,2-benzisoselenazolone 3(2*H*)-ketone)] ethane, BBSKE), a promising organoselenium anticancer agent with low toxicity in animal models, has been developed by our group and is currently undergoing phase I clinical trials [24–29]. Its anticancer effectiveness has been proven in various human cancer cell lines and tumor-bearing mouse models [24–27,29,30]. Interestingly, the inhibition of cancer cell growth by ethaselen is correlated with TrxR1 inactivation in various investigated cell lines, including HeLa (human cervical cancer), BGC823 (human stomach adenocarcinoma), HL60 and K562 (human leukemia), A549 (human lung cancer), LoVo (human colon cancer), Bel-7402 (human epithelial hepatoma), Tca8113 (human tongue cancer), and KB (human nasopharyngeal epidermal carcinoma) cells [24–27]. In addition, ethaselen markedly suppresses tumor growth in human A549-grafted nude mice along with TrxR1 inactivation in tumor tissue [30]. However, the mechanism of action and the inhibitory properties of ethaselen on mammalian TrxR1 and its impact on the intracellular TrxR/Trx redox system remain to be elucidated in detail.

In this work, we provide evidence that ethaselen targets mammalian TrxR1 at the C-terminal active site acting as a potent inhibitor and a rather weak substrate of the enzyme. Disruption of the TrxR-dependent reduction of oxidized Trx by ethaselen leads to the accumulation of oxidized Trx and reactive oxygen species in A549 cells. Thus ethaselen, as a selenium-containing anticancer agent, can be added to the diversity of known mammalian TrxR inhibitors.

Materials and methods

Enzymes and reagents

Rat liver TrxR1, *Escherichia coli* TrxR and Trx, bovine insulin, reduced and oxidized glutathione, NADPH, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), iodoacetic acid, and 2′,7′-dichlorfluorescein diacetate (DCFH-DA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Human placental TrxR1, the recombinant Sec498Cys TrxR1 mutant (Sec → Cys), and the truncated TrxR1 missing the last two amino acids (Sec–Gly), as well as human GR and the human Trx mutant Cys73 → Ser (which does not form dimers and is stable in enzymatic assays), were produced as described before [31–34]. Ethaselen was synthesized in our group and dissolved in a stock solution of dimethyl sulfoxide (DMSO) as described [24]. All other reagents were of analytical grade.

Molecular docking and dynamics

The mammalian TrxR1 structure was obtained from the Protein Data Bank (PDB ID: 1H6V) [35]. A 1000-ps molecular dynamics (MD) simulation of the complex was done with an Amber 8 molecular simulation package [36]. The initial conformation was taken from the lowest binding energy docking conformation. It was then solvated in TIP3P water using an octahedral box, which was extended 8 Å away from any solute atom. The solvated system was neutralized by adding a sodium ion into the simulation. Molecular dynamics simulation was carried out using the SANDER (simulated annealing with NMR-derived energy restraints) module of Amber 8. The solvent (water molecules) was optimized with 250 steps of steepest descent followed by 750 steps of conjugate gradient, with a large constraint of 500 kcal mol^{-1} $Å^{-2}$ on solute (protein, counter ion, ligand) atoms. The full system was minimized using the steepest decent and conjugated gradient methods consecutively without a force constraint. After minimization, 100 ps of MD were done for the complex, during which the temperature was slowly increased from 0 to 300K. The production simulation of 1000 ps was followed at constant pressure (1 atm) and temperature (300K). SHAKE was applied to all hydrogen atoms in the entire simulation. A cutoff of 12 Å was used for Lennard–Jones interactions. The final structure of the complex of TrxR1 and ethaselen for future analysis was produced from the 1000 steps of the minimized and averaged structure of the last 500 ps of MD.

Enzyme assays

All enzyme activity assays and kinetic studies were carried out in at least three independent experiments at room temperature using a Hitachi U-2001 spectrophotometer.

Both DTNB and Trx reduction assays were used to measure TrxR activity [37,38]. Mammalian TrxRs were prereduced by incubation with NADPH for 5 min. Then reduced human placental TrxR1 (4 nM) and rat TrxR (0.15 μM) were incubated with different concentrations of ethaselen in 490 μl of 0.1 M phosphate buffer (200 μM NADPH, 1 mM EDTA, pH 7.5) and for different incubation times at room temperature in the dark. For the DTNB reduction assay, 10 μl of 0.1 M phosphate buffer containing DTNB (final concentration: 2 mM) was added. The increase in absorbance at 412 nm was recorded in the initial 3 min. Likewise the activities of the recombinant human TrxR1 variants (Sec498 → Cys TrxR1 mutant and Sec–Gly truncated TrxR1 mutant) in the presence of ethaselen were determined (concentrations of ethaselen up to 120 μM). For the Trx reduction assay, 10 μM human recombinant Trx was added, followed by monitoring the initial decrease in absorbance at 340 nm.

To investigate the inhibition kinetics of ethaselen on mammalian TrxR1, various concentrations of the disulfide substrate DTNB (31.25–2000 μM) and ethaselen (0–46.28 nM) were used [39]. After the simultaneous addition of DTNB and ethaselen into the reaction buffer containing 4 nM human TrxR1 and 200 μM NADPH, the increase in absorbance at 412 nm was recorded in the initial 60 s (the enzymatic reaction is linear during this period). The inhibition of human TrxR1 by ethaselen was analyzed using Lineweaver–Burk plots [39]. The inhibition constants K_i and K_{is} (equilibrium constants for the inhibitor binding to free enzyme and to the enzyme–substrate complex) were obtained by creating secondary plots of either the slope or the intercept of the primary plot against the concentration of ethaselen [40].

To study if ethaselen can be reduced by TrxR1, we adapted a DTNB-coupled assay to spectrophotometrically detect one of the two possible reduced forms (Scheme 1). DTNB is a sensitive and rapid thiol/selenothiol-detecting reagent. For the experimental design, low and high concentrations of ethaselen (8, 64, 100 μM) and human TrxR1 (4, 20, 40 nM), covering the same concentrations used in the experiments for Fig. 3, were employed. Ethaselen and human TrxR1 were incubated in 0.1 M phosphate buffer (1 mM EDTA, 200 μM NADPH, pH 7.4) at room temperature for various times (15, 30, and 60 min) in the dark. After the removal of human TrxR1 via a molecular weight cut-off 3000 filter (Vivaspin), DTNB