Free Radical Biology and Medicine 63 (2013) 485–494

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

A diterpenoid derivate compound targets selenocysteine of thioredoxin reductases and induces Bax/Bak-independent apoptosis

Jinhua Liu [a], Chenglong Mu [a], Wen Yue [b], Jie Li [a], Biao Ma [a], Lixia Zhao [b], Lei Liu [b], Quan Chen [a,b], Chen Yan [c], Haiyang Liu [c], Xiaojiang Hao [c], Yushan Zhu [a,*]

[a] Tianjin Key Laboratory of Protein Science, College of Life Sciences, Nankai University, Tianjin 300071, China
[b] State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[c] The State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China

ARTICLE INFO

Article history:
Received 28 March 2013
Received in revised form
23 May 2013
Accepted 24 May 2013
Available online 31 May 2013

Keywords::
Thioredoxin reductase
Bim
ROS
Apoptosis
Cancer

ABSTRACT

We have previously shown that the natural diterpenoid derivative S3 induced Bim upregulation and apoptosis in a Bax/Bak-independent manner. However, the exact molecular target(s) of S3 and the mechanism controlling Bim upregulation are still not clear. Here, we identify that S3 targets the selenoproteins TrxR1 and TrxR2 at the selenocysteine residue of the reactive center of the enzymes and inhibits their antioxidant activities. Consequently, cellular ROS is elevated, leading to the activation of FOXO3a, which contributes to Bim upregulation in Bax/Bak-deficient cells. Moreover, S3 retards tumor growth in subcutaneous xenograft tumors by inhibiting TrxR activity *in vivo*. Our studies delineate the signaling pathway controlling Bim upregulation, which results in Bax/Bak-independent apoptosis and provide evidence that the compounds can act as anticancer agents based on mammalian TrxRs inhibition.

Introduction

Redox homeostasis is crucial for cellular viability and function [1–4], the balance of which is maintained by two major cellular antioxidant systems, the glutathione system and the thioredoxin (Trx) system [5]. The Trx system, which consists of thioredoxin, thioredoxin reductase (TrxR), and nicotinamide adenine dinucleotide phosphate (NADPH), plays important roles in antioxidant defense and redox-regulated signal transduction for cell growth and apoptosis [6–9]. Three TrxRs, cytosolic TrxR1, mitochondrial TrxR2, and testis-specific TrxR3, have been identified in mammalian cells [10–13]. TrxRs are essential mammalian selenoproteins that act as homodimers to catalyze NADPH-dependent reduction of Trx and other small molecular oxidants [14,15]. The fully oxidized enzyme accepts electrons from NADPH by reducing the bound flavin adenine dinucleotide (FAD) to flavin adenine dinucleotide reduced (FADH2), which passes the reducing equivalents to the N-terminal redox-active center in one subunit. This reduced N-terminal redox center transfers two electrons to the C-terminal redox-active selenocysteine (Sec) site in another subunit to generate the active enzyme [16–18]. The conserved Sec is essentially involved in the catalytic cycle of TrxR and is able to easily interact with its substrates and inhibitors according to the low pK_a (5.3) value of Selenol [16,17,19].

Both Trx and TrxR have important functions in the neoplastic growth of cancer cells and are key components of the resistant phenotype [20]. TrxR and components of Trx systems function as signaling factors that use critical cysteine motif(s) to act as redox-sensitive "sulfhydryl switches." These switches reversibly modulate specific signal transduction cascades, which regulate downstream proteins with similar redox-sensitive sites [21,22], and they are able to maintain critical cysteine residues present in transcription factors in a reduced state, which allows DNA binding. These transcription factors include nuclear transcription factor κ (NF-κB), the tumor suppressor p53 [23], the glucocorticoid receptor [24], hypoxia-inducible factor 1α (HIF-1α) [25,26], and the activator protein-1 (AP1) protein complex [21]. Both cytosolic and mitochondrial TrxR genes are essential for embryonic development, and knocked out these genes result in embryonic lethality [27–29]. Trx and TrxR have also been reported to be highly expressed in a

Abbreviations: AP1, activator protein-1; ASK1, apoptosis signal-regulating Ćkinase 1; CBB, Coomassie brilliant blue; DMEM, Dulbecco's modified Eagle's medium; DNCB, dinitrochlorobenzene; DTNB, dithiobisnitrobenzoic acid; GRs, glutathione reductases; HIF-1α, hypoxia-inducible factor 1α; MEF, mouse embryonic fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-l-cysteine; NF-κB, nuclear transcription factor κ; PI, propidium iodide; ROS, reactive oxygen species; Sec, selenocysteine; Trx, thioredoxin; TrxR, thioredoxin reductase; S3, 15-oxospiramilactone.

* Corresponding author.
E-mail address: zhuys@nankai.edu.cn (Y. Zhu).

0891-5849/$ - see front matter Crown Copyright © 2013 Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2013.05.038

variety of cancers, including lung, colorectal, and pancreatic cancer [30–32], and increased expression of TrxR has been correlated with aggressive tumor growth, multiple drug resistance, poorer prognosis, and decreased patient survival [30,33]. Therefore, inhibition of TrxRs has been regarded as an important strategy for anticancer therapy [34,35]. This inhibition leads to p53-dependent cell cycle arrest or apoptosis [36] or Trx-dependent activation of apoptosis signal-regulating kinase 1 (ASK1) [37]. Alternatively, inhibition of TrxR hinders electron flux through the Trx system to change the redox conditions of cells, leading to the alteration of cellular signaling systems, and triggers apoptosis and necrosis due to reactive oxygen species (ROS) accumulation [38,39].

After exposing to apoptosis stress, the Bcl-2 family protein Bax and Bak induce the mitochondrial outer membrane permeabilization (MOMP) for the release of cytochrome *c* and lead the cells to undergo programmed cell death. Bax and Bak share a redundant function to initiate apoptosis in response to damaged cells or disorganized proliferation, especially in tumorigenesis [40,41]. However, in many kinds of tumors, the functions of Bax and Bak are downregulated through different ways. For example, Bax mutation has been identified in human leukemia, colorectal cancer, and gastrointestinal cancer [42,43]. Bak expression has also been abolished in skin cancer and gastric and colorectal cancers [44]. Therefore, innovative and specific approaches to overcome survival of the tumor cells in the absence of Bax and Bak function are urgently required. We previously found that natural diterpenoid derivative, 15-oxospiramilactone (hereafter named S3), induces Bax/Bak-independent apoptosis through the upregulation of a Bcl-2-interacting mediator of cell death (Bim), which interacts with Bcl-2 to permeabilize the mitochondrial outer membrane for cytochrome *c* release and apoptosis [45]. In an effort to understand the molecular targets of the compounds involved in the upregulation of Bim, we took a chemical biology approach and identified that S3 could target TrxR1 and TrxR2 and inhibited their antioxidant activities. Elucidation of the molecular signaling pathway controlling Bim upregulation by a small natural compound may hold promise for therapeutic applications of anticancer agents.

Materials and methods

Cell lines and reagents

SV40 T antigen-immortalized *bax*$^{-/-}$/*bak*$^{-/-}$ mouse embryonic fibroblast (MEF) cells and HCT116 *bax*$^{-/-}$ cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin–streptomycin at 37°C and 5% CO_2. Antibodies against the following proteins were used: β-actin (A5441, Sigma), TrxR1 (ab16840, abcam), c-Myc and TrxR2 (Sc-40, Sc-46279, Santa Cruz), Bim (C3405, CST), FOXO3a (2497, CST), p-FOXO3a (9465, CST), and Biotin (D5A7, CST). Commercial rat liver TrxR was purchased from Sigma (T9698). Other chemicals were purchased from Sigma unless otherwise specified.

TrxR1 and TrxR2 plasmid construction and protein purification

Full-length human TrxR1 (NM_001093771.2) and TrxR2 (NM_006440.3) and mouse TrxR1 (NM_001042513.1) and TrxR2 (NM_013711.3) cDNAs were amplified by PCR and cloned into the pcDNA4TO-myc vector. Mouse TrxR1 (Sec498A) and TrxR2 (Sec498A) site-directed mutant constructs were made using the site-directed mutagenesis kit. TrxR1 (Sec498C; Sec498A) and TrxR2 (Sec498C; Sec498A) were digested from the pcDNA4TO vector with EcoRI and XhoI, and the fragments were then ligated into pGEX-4 T-1 vectors and expressed in *E. coli* BL21 cells. Protein expression was induced by adding 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified by glutathione-Sepharose columns (Roche). The purity of the proteins was assessed by SDS-PAGE followed by Coomassie brilliant blue (CBB) staining [46].

Detection of apoptotic activities

Cells were treated with 10 μM S3 or S1 (Fig. S1A and B) for the indicated time periods, and apoptosis was measured using the Annexin V detection kit according to the manufacturer's instructions. Flow cytometric analysis was performed to monitor the green fluorescence of FITC-conjugated Annexin V and the red fluorescence of DNA-bound propidium iodide (PI). All data were analyzed with Cell Quest software (BD).

Determination of TrxR activity in vivo and in vitro

For *in vivo* TrxR activity, *bax*$^{-/-}$/*bak*$^{-/-}$ MEFs were incubated with different concentrations (5, 10, and 20 μM) of S3 or S1 for 12 and 24 h. The control group contained the same amount of DMSO (1%, v/v). Next, the cells were washed with phosphate-buffered saline once and lysed with cell lysis buffer (0.5% NP-40, 150 mM NaCl, and 1 mM EDTA in TE buffer) with a protease inhibitor cocktail (Roche). The activity of TrxR in the cell extracts was determined as previously described [22].

For *in vitro* TrxR activity, commercial purified rat liver TrxR (Sigma) was first reduced by incubation with excess NADPH at room temperature for 5 min in buffer containing 50 mM Tris-Cl and 1 mM EDTA (pH 7.5). Next, appropriate amounts of S3 were added, followed by incubation at room temperature for the appropriate time. The control group contained the same amount of DMSO, and equal amounts of TrxR were subjected to the dithiobisnitrobenzoic acid (DTNB) assay as described above.

Binding kinetics of TrxR and Biotin–S3

Commercial rat liver TrxR1 (Sigma) was incubated with 40 μM Biotin–S3 for different time periods, and the covalently linked TrxR–Biotin–S3 complexes [TrxR–S3] were analyzed by SDS gel and subsequent Western blotting with anti-Biotin antibody. The protein bands on the gels were quantified by densitometry. Gel photographic images were processed using Image-J software.

The reaction rate constant of k_{obs} follows as the equations according to reference [47,48]:

$$\mathrm{TrxR} + \mathrm{S3} \rightarrow \mathrm{TrxR\text{–}S3}$$

$$[\mathrm{TrxR}]_t = [\mathrm{TrxR}]_0 * E^{-\mathrm{kobs}*t} \quad (1)$$

$$[\mathrm{TrxR\text{–}S3}] = [\mathrm{TrxR}]_0 - [\mathrm{TrxR}]_t = [\mathrm{TrxR}]_0 * (1 - E^{-\mathrm{kobs}*t}), \quad (2)$$

where [TrxR–S3] is the complex of TrxR with S3, $[\mathrm{TrxR}]_0$ is the total concentration, and $[\mathrm{TrxR}]_t$ is TrxR concentration at time *t*.

Subcutaneous tumor implantation model

E1A/K-Ras-transformed *bax*$^{-/-}$/*bak*$^{-/-}$MEFs and HCT116 *bax*$^{-/-}$ cells were harvested by centrifugation and suspended in PBS. A tumor cell suspension (5×10^5 *bax*$^{-/-}$/*bak*$^{-/-}$MEFs or 3×10^6 HCT116 *bax*$^{-/-}$ cells in 100 μl PBS, respectively) was injected into the underarm of 4- to 5-week-old nude BALB/c mice, and then the mice were separated into three groups (6 mouse/group). Seven days after implantation, animals were intraperitoneally treated with S3 (30 mg/kg/day) or S1 (30 mg/kg/day) or vehicle control (50% (v/v) propylene glycol in 1.8% NaCl) every 3 days for 3 weeks.