Free Radical Biology and Medicine 89 (2015) 192–200

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Oxidation of structural cysteine residues in thioredoxin 1 by aromatic arsenicals enhances cancer cell cytotoxicity caused by the inhibition of thioredoxin reductase 1 ☆

Xu Zhang [a], Jun Lu [a,*], Xiaoyuan Ren [a], Yatao Du [a], Yujuan Zheng [b], Panayiotis V. Ioannou [c], Arne Holmgren [a,*]

[a] Division of Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77, Stockholm, Sweden
[b] Department of Oncology and Pathology, Cancer Centrum Karolinska, Karolinska Institutet, SE 171 76 Stockholm, Sweden
[c] Department of Chemistry, University of Patras, Rio 26500, Greece

ARTICLE INFO

Article history:
Received 11 February 2015
Received in revised form
18 June 2015
Accepted 7 July 2015
Available online 11 July 2015

Keywords:
Thioredoxin
Thioredoxin reductase
Arsenical
Oxidative stress
Cytotoxicity

ABSTRACT

Thioredoxin systems, composed of thioredoxin reductase (TrxR), thioredoxin (Trx) and NADPH, play important roles in maintaining cellular redox homeostasis and redox signaling. Recently the cytosolic Trx1 system has been shown to be a cellular target of arsenic containing compounds. To elucidate the relationship of the structure of arsenic compounds with their ability of inhibiting TrxR1 and Trx1, and cytotoxicity, we have investigated the reaction of Trx1 system with seven arsenic trithiolates: As(Cys)$_3$, As(GS)$_3$, As(Penicillamine)$_3$, As(Mercaptoethanesulfonate)$_3$, As(Mercaptopurine)$_3$, As(2-mercaptopyridine)$_3$ and As(2-mercaptopyridine N-oxide)$_3$. The cytotoxicity of these arsenicals was consistent with their ability to inhibit TrxR1 *in vitro* and in cells. Unlike other arsenicals, As(Mercaptopurine)$_3$ which did not show inhibitory effects on TrxR1 had very weak cytotoxicity, indicating that TrxR1 is a reliable drug target for arsenicals. Moreover, the two aromatic compounds As(2-mercaptopyridine)$_3$ and As(2-mercaptopyridine N-oxide)$_3$ showed stronger cytotoxicity than the others. As(2-mercaptopyridine)$_3$ which selectively oxidized two structural cysteines (Cys62 and Cys69) in Trx1 showed mild improvement in cytotoxicity. As(2-mercaptopyridine N-oxide)$_3$ oxidized all the Cys residues in Trx1, exhibiting the strongest cytotoxicity. Oxidation of Trx1 by As(2-mercaptopyridine)$_3$ and As(2-mercaptopyridine N-oxide)$_3$ affected electron transfer from NADPH and TrxR1 to peroxiredoxin 1 (Prx1), which could result in the reactive oxygen species elevation and trigger cell death process. These results suggest that oxidation of structural cysteine residues in Trx1 by aromatic group in TrxR1-targeting drugs may sensitize tumor cells to cell death, providing a novel approach to regulate cellular redox signaling and also a basis for rational design of new anticancer agents.

1. Introduction

Arsenic is a naturally occurring metalloid, which widely exists in soil, water and air. Exposure to arsenic may cause acute poisoning or chronic illness and several diseases including cancer [1,2]. Paradoxically, arsenic trioxide (ATO) has been used in the treatment of cancer [3]. After it was first discovered as a treatment for acute promyelocytic leukemia, many following studies also raised the possibility of using ATO in solid cancers [4]. Besides of ATO, currently over 100 arsenic compounds including inorganic and organic arsenicals have been used in the clinical trials for the treatment of cancer [5]. However, the relationship of the structure and function is not clear. Clarification of this relation is helpful for the evaluation of efficiency of the arsenicals and the design of new anticancer compounds.

Arsenicals can kill cancer cells through several mechanisms [6],

Abbreviations: **As1**, As(Cys)$_3$; **As2**, As(GS)$_3$; **As3**, As(Penicilliamine)$_3$; **As4**, As(SCH$_2$CH$_2$SO$_3$Na)$_3$; **As5**, As(Mercaptopurine)$_3$; **As6**, As(2-mercaptopyridine)$_3$; **As7**, As(2-mercaptopyridine N-oxide)$_3$; **ATO**, arsenic trioxide; ASK1, apoptosis signal-regulating kinase-1; Cys, cysteine; CBB, Coomassie Brilliant Blue R250; DMEM, Dulbecco´s modified Engle´s medium; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, Dithiothreitol; EGTA, ethylene glycol tetraacetic acid; FAD, flavin adenine dinucleotide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; Grx, glutaredoxin; IAA, iodoacetic acid; IAM, iodoacetamide; MOPS, 3-(N-morpholino)propanesulfonic acid; MTT, thiazolyl blue tetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate; Prx, peroxiredoxin; ROS, reactive oxygen species; Sec, selenocysteine; TNB, 5-thio-2-nitrobenzoic acid; Trx, thioredoxin; TrxR, thioredoxin reductase

☆The authors declare no competing financial interest.

* Corresponding authors. Fax: +46 8 305193.
E-mail addresses: grace.zhangxu@gmail.com (X. Zhang), Jun.lu@ki.se (J. Lu), xiaoyuan.ren@ki.se (X. Ren), yataodu@gmail.com (Y. Du), yujuan.zheng@ki.se (Y. Zheng), ioannou@chemistry.upatras.gr (P.V. Ioannou), arne.holmgren@ki.se (A. Holmgren).

http://dx.doi.org/10.1016/j.freeradbiomed.2015.07.010
0891-5849/

including interruption of the redox balance. Arsenicals can inhibit mitochondrial respiratory function, and increase ROS generation [7]. Additionally, arsenicals like ATO can react with sulfhydryl groups, such as in glutathione (GSH) [8,9], or enzyme active sites such as the selenocysteine (Sec) in thioredoxin reductase (TrxR) [10], leading to the inactivation of antioxidant enzymes and cell apoptosis in cancer cells. The thioredoxin system, which is composed of nicotinamide adenine dinucleotide phosphate (NADPH), thioredoxin (Trx) and TrxR, is one of the major disulfide reductase system in the cells [11]. There are two distinct Trx systems in mammalian cells: Trx1 system mainly in cytosol and nucleus and Trx2 system in mitochondrial matrix [12]. Trx1 system has a wide range of substrates, such as ribonucleotide reductases, methionine sulfoxide reductases and peroxiredoxins (Prxs). Prxs are at the forefront of defending against oxidative stress by eliminating excess hydrogen peroxide (H_2O_2) in living cells. Through these substrates, Trx1 plays important roles in DNA synthesis and cellular redox maintaining. In addition, Trx1 is also known to regulate several transcription factors such as NF-κB, p53 and Ref-1, as well as some apoptosis factors like apoptosis signal-regulating kinase 1 (ASK1) [12–16]. Trx1 redox state and TrxR1 activity are crucial factors for cell fate, especially for cancer cells since Trx1 and TrxR1 are overexpressed to balance the elevated reactive oxygen species (ROS) in many cancer cells [12,14,16,17].

Trx1 has two cysteine residues in its active site (WCGPC), which can reduce the disulfide of target proteins by a thiol-disulfide exchange reaction. In turn, the oxidized disulfide in Trx1 can be reduced by TrxR1 and NADPH [11]. Unlike Trx2, which has only two cysteine residues in its active site, Trx1 has three additional structural cysteine residues (cysteine 62, cysteine 69 and cysteine 73). These structural cysteine residues, especially Cys62 and Cys69, which can form an extra disulfide in Trx1, attract more and more interests today. Accumulating evidence has shown that the extra disulfide formed between Cys62 and Cys69 is important for the regulation of Trx1 activity and have implications for Trx1 cellular functions in redox signaling by H_2O_2 [18,19].

TrxR1, a key seleno-enzyme, has been found to be a primary target of ATO and redox states of Trx1 can be influenced eventually in the ATO-induced apoptotic cells, which has been proposed to be a cancer therapeutic basis of ATO [10]. To evaluate the relationship of the function and structure of arsenicals, seven organo-arsenicals (Fig. 1) have been used in this study on the inhibition of TrxR1, Trx1 and cell toxicity. Our results suggest that TrxR1 is a reliable target of arsenical treatment, and the inhibition of TrxR1 is required for arsenicals to exert their cytotoxicity. Two arsenicals containing aromatic groups, As6 and As7, were found to be able to react and oxidize the thiols in structural Cys in Trx1. The oxidation of Trx1 by As6 and As7 facilitated the inhibitory effects on Trx1 activity to reduce Prx1 and enhanced cell toxicity.

2. Material and methods

2.1. Chemicals and Proteins

Human wild-type Trx1 and mutant Trx1 (C61S/C72S), Trx2, Prx1, goat anti-Trx1, and anti-rat TrxR1 antibodies were from IMCO Ltd. (Stockholm, Sweden; www.imcocorp.se). Anti-Trx2, anti-heme oxygenase 1 and GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibody to TrxR2 was from Acris Antibodies, Inc. (San Diego, CA, USA). Preparation of recombinant rat TrxR1 was described previously [20]. **As 1-7** (Fig. 1) were synthesized according to literature: **As1**-**3** were synthesized according to reference [9]; **As4** and **As5** were synthesized according to reference [21]; **As6** was synthesized according to reference [22]; and **As7** was synthesized according to reference [23,24]. The purity of the compounds was better than 99% and were characterized by melting point, elemental analyses, IR, NMR and in the case of **As7** by X-ray analysis [24]. The compounds were stable in water due to the high affinity of arsenic to sulfur. After the compounds were synthesized at the University of Patras, Greece, they were shipped to Stockholm. **ATO** and other chemicals were from Sigma. To prepare the stock solutions, **ATO** and **As1** were dissolved in 1 N NaOH, **As2-4** were dissolved in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and **As5-7** were dissolved in ethanol.

2.2. Inhibition of TrxR1 by arsenicals in vitro

NADPH-reduced TrxR1 (1 μM) was incubated with arsenic compounds at room temperature for 60 min. Then, TrxR1 activity was assayed by insulin reduction assay in the solution containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 μM NADPH, in the presence of 5 μM Trx1 and 160 μM insulin. The absorbance at 340 nm was followed with a VERSA microplate reader (Molecular Devices, Sunnyvale, CA, USA). TrxR1 activity was calcuated by measuring the slope of absorbance change during the initial 10 minutes.

2.3. Cell Culture

Human neuroblastoma SH-SH5Y cells were cultured with 1 g/L glucose Dulbecco's modified Eagle's medium (VWR) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% (v/v) fetal calf serum at 37 °C in an incubator with 5% CO_2.

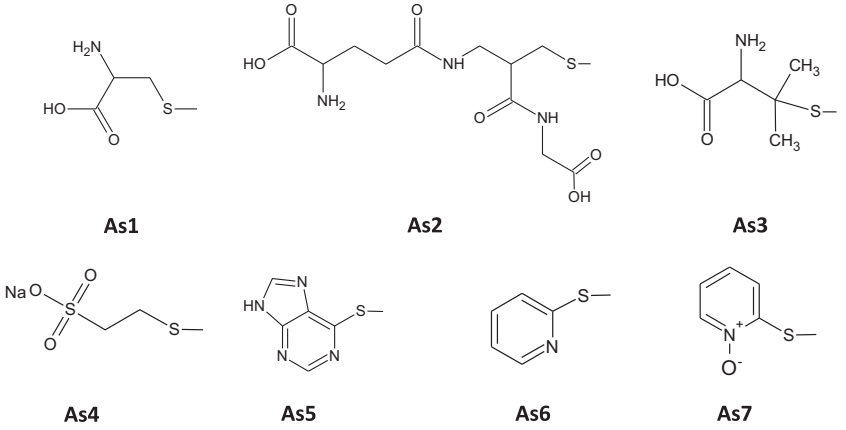

Fig. 1. R group of seven arsenothiols (AsR_3) which were synthesized and used in this study: **As1**, $As(Cys)_3$; **As2**, $As(GS)_3$; **As3**, $As(Penicillamine)_3$; **As4**, $As(SCH_2CH_2SO_3Na)_3$; **As5**, $As(Mercaptopurine)_3$; **As6**, $As(2\text{-mercaptopyridine})_3$; and **As7**, $As(2\text{-mercaptopyridine N-oxide})_3$.