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Inhibition of thioredoxin reductase by alantolactone prompts oxidative stress-mediated apoptosis of HeLa cells

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ABSTRACT

The mammalian thioredoxin reductase (TrxR) isoenzymes, TrxR1 in cytosol or nucleus, TrxR2 in mitochondria, and TrxR3 in testis, are essential seleno-flavoenzymes with a conserved penultimate selenocysteine (Sec) residue at the C-terminus, and have attracted increasing interests as potential targets for development of cancer chemotherapeutic agents. The sesquiterpene lactone alantolactone (ATL), an active component from the traditional folk medicine *Inula helenium*, has been documented possessing multiple pharmacological functions, especially the anticancer activity. However, the underlying mechanism has not been well defined. We reported that ATL inhibits both the recombinant TrxR and the enzyme in the cellular environment. The alpha-methylene-gamma-lactone moiety in ATL and the Sec residue in TrxR are critical for targeting TrxR by ATL. By employing our newly developed pull down assay, we demonstrated the remarkable elevation of the oxidized thioredoxin in HeLa cells after ATL treatment. In addition, ATL elicits accumulation of reactive oxygen species, and eventually induces apoptosis of HeLa cells. Importantly, overexpression of the functional TrxR attenuates the cytotoxicity of ATL, while knockdown of the enzyme sensitizes the cells to ATL treatment. Targeting TrxR thus discloses a novel molecular mechanism underlying the cellular action of ATL, and sheds light in considering the usage of ATL as a potential cancer chemotherapeutic agent.

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1. Introduction

Plant-derived natural products have provided and continue to provide a rich and unique source of therapeutic agents [1,2]. Sesquiterpene lactones are a large and diverse family of natural products widely present in a variety of herbal plants, and display multiple pharmacological functions [3,4]. Alantolactone (ATL, Fig. 1), a sesquiterpene lactone containing an α-methylene-γ-lactone moiety, is the principal active component from *Inula helenium*, a traditional medicinal herb that has been extensively used to treat diverse ailments for centuries [5]. The presence of ATL in *I. helenium*, especially in the root, is highly abundant (>2% of the dry root) [6,7]. ATL has been documented having antibacterial activity [8], targeting Nrf2 to activate detoxifying enzymes [9], and possessing anti-inflammatory property by suppressing inducible nitric oxide synthase and cyclooxygenase-2 [10] or inhibiting chemokine production [11]. In recent years, increasing interests have been attracted by the potential anticancer activity of ATL in different types of tumor cells, and the putative mechanisms have been reported, including arrest of cell cycles [12], suppression of STAT3 activation [13], downregulation of testes-specific protease 50 [14], induction of reactive oxygen species (ROS) [15], inhibition of Bcr/Abl [16], and modulation of activin/SMAD3 signaling [17], and others [7]. Despite the potent anticancer efficacy of ATL, its molecular mechanism is still elusive, and the primary cellular target and mode of action of this small molecule remain unclear.

The mammalian thioredoxin reductase (TrxR) isoforms, including the cytosolic/nuclear enzyme TrxR1, the mitochondrial enzyme TrxR2, and the testis-specific isoform TrxR3, are essential selenocysteine (Sec)-containing flavoenzymes with an indispensable Sec residue at the penultimate C-terminal position. TrxR, thioredoxin (Trx) and NADPH comprise the thioredoxin system, which is a highly conserved and ubiquitous system and plays pivotal roles in maintaining intracellular redox homeostasis [18–20]. TrxRs catalyze the transfer of electrons from NADPH to the active site of Trxs generating the reduced Trxs, which interacts with a panel of downstream target proteins ranging from metabolic enzymes to kinases to transcription factors, to regulate a wide array of cellular redox events involved in cell proliferation, differentiation and death [21,22]. Expanding evidence has

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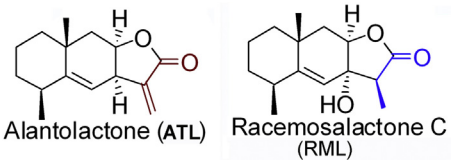

Fig. 1. Chemical structures of ATL and RML. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

implicated that the malfunction of the system is a causal, or at least an ancillary, factor of a number of diseases, such as cancer, cardiovascular diseases and neurodegenerative disorders [22,23]. TrxRs are the only enzymes known to catalyze the reduction of Trxs under physiological conditions, and hence the function of the thioredoxin system is tightly regulated by the activity of the selenoenzymes. The thioredoxin system is often upregulated in many malignant cells [24–26]. Targeting Trx/TrxR ablation leads to a retardation in tumor progression and metastasis [27,28], while overexpressing Trx inhibits apoptosis and promotes tumor angiogenesis [29–31]. These observations suggest that the thioredoxin system is critically involved in tumorigenesis and a potential target for cancer treatment. Thus, the growing attention has been paid to identify and develop small molecules targeting the TrxR as potential cancer chemotherapeutic agents [32–42].

As part of our continuing interests in discovering and developing novel small molecule regulators of cellular redox systems [32,40–44], we reported herein that ATL, via its α-methylene-γ-lactone moiety, inhibits the recombinant TrxR1 by targeting the Sec residue in vitro. ATL also causes TrxR inhibition, ROS production and oxidized Trx accumulation in HeLa cells, and eventually induces apoptosis of HeLa cells. Importantly, overexpression of functional TrxR1 significantly alleviates the cytotoxicity of ATL, while knockdown of TrxR1 augments the cytotoxicity, supporting the physiological significance of targeting TrxR by ATL. Targeting TrxR thus discloses an unprecedented mechanism underlying the cellular action of ATL, and sheds lights in understanding the anticancer function of ATL and developing ATL as a cancer therapeutic agent.

2. Materials and methods

2.1. Chemicals and enzymes

Recombinant rat TrxR1 (WT TrxR1) was essentially prepared as described [45], and is a gift from Prof. Arne Holmgren at Karolinska Institute, Sweden. The recombinant U498C TrxR1 mutant (Sec → Cys) was produced as described [40]. Proteins were pure as judged by Coomassie-stained SDS-polyacrylamide gel electrophoresis (PAGE), and the recombinant TrxR1 had a specific activity of 50% of the wild type TrxR1 with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) assay. The recombinant *Escherichia coli* Trx was purchased from IMCO (Stockholm, Sweden, www.imcocorp.se). Dulbecco's modified Eagle's medium (DMEM), G418, *N*-acetyl-L-cysteine (NAC), bovine insulin, L-buthionine-(*S*,*R*)-sulfoximine (BSO), *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA), reduced glutathione (GSH), dimethyl sulfoxide (DMSO), sodium 2,3-dimercapto-1-propanesulfonic acid (DMPS), Hoechst 33342, puromycin, DL-dithiothreitol (DTT), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 2′,7′-dichlorfluorescein diacetate (DCFH-DA) were obtained from Sigma–Aldrich (St. Louis, USA). NADPH was obtained from Roche (Mannheim, Germany). Fetal bovine serum (FBS) was obtained from Sijiqing (Hangzhou, China). Anti-Trx1 and anti-TrxR1 antibodies and dihydroethidium (DHE) were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). The PVDF membrane was from Millipore (Billerica, USA). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), penicillin and streptomycin were obtained from Amresco (Solon, USA). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (V) (Na_3VO_4) and anti-actin antibody were obtained from Beyotime (Nantong, China). DTNB was obtained from J&K Scientific (Beijing, China). The apoptosis detection kit containing fluorescein-5-isothiocyanate-conjugated Annexin V (Annexin V-FITC) and propidium iodide (PI) was from Zoman Biotech (Beijing, China). Sephadex G-25 was from GE Healthcare. shRNA plasmids targeting TrxR1 (shTrxR1) and non-targeting control (shNT), and HEK cells stably overexpressing TrxR1 (HEK-TrxR1) and the cells stably transfected with a vector (HEK-IRES) were gifts from Prof. Constantinos Koumenis from University of Pennsylvania School of Medicine [46,47]. ATL and racemosalactone C (RML) were isolated from the roots of *I. racemosaas* described in our previous publication [48]. A 50 mM solution of ATL was prepared in DMSO and stored at −20 °C, and the final concentrations of DMSO are no more than 0.1% (V/V) in experiments unless otherwise noted. All other reagents were of analytic grade.

2.2. Cell cultures

HeLa cells, HL-60 cells, Hep G2 cells and HEK 293T cells, obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, were cultured in the DMEM supplemented with 10% FBS, 2 mM glutamine, and 100 units/mL penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. HeLa-shNT and HeLa-shTrxR1 cells were generated in our lab [41,42] and were cultured under the same conditions as those of HeLa cells with additional supplement of puromycin (1 μg/mL) in the culture medium. HEK-TrxR1 and HEK-IRES cells were cultured in DMEM with 10% FBS, 2 mM glutamine, 100 units/mL penicillin/streptomycin, 0.1 μM sodium selenite, and 0.4 mg mL^{-1} G418 and maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Cell viability assay

The cell viability was measured using the MTT assay. Unless otherwise noted, 5×10^3 cells were incubated with ATL or other agents in triplicate in a 96-well plate for indicated times at 37 °C in a final volume of 100 μL. Cells treated with DMSO alone were used as controls. At the end of the treatment, 10 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C. An extraction buffer (100 μL, 10% SDS, 5% iso-butanol, 0.1% HCl) was added, and the cells were incubated overnight at 37 °C. The absorbance of control cells (cells with vehicle treatment, $A_{Control}$), ATL-treated cells (A_{ATL}) and blank sample (cell culture medium, MTT and exaction buffer, A_{Blank}) was measured at 570 nm using a microplate reader (Thermo Scientific Multiskan GO, Finland). The viability was calculated by the following formula: (cell viability) % = $(A_{ATL} - A_{Blank})/(A_{Control} - A_{Blank}) \times 100$.

2.4. In vitro TrxR activity assays

The TrxR activity was determined by the DTNB reduction assay [41,42]. The NADPH-reduced TrxR (170 nM) or U498C TrxR (700 nM) was incubated with different concentrations of ATL for the indicated times at room temperature (the final volume of the mixture was 50 μL) in a 96-well plate. A master mixture (50 μL) containing DTNB and NADPH in TE buffer (50 mM Tris–HCl pH 7.5, 1 mM EDTA) was added. The final concentrations of DTNB and NADPH in the assay mixture are 2 mM and 200 μM, respectively. The linear increase of absorbance at 412 nm during the initial 3 min was recorded. The same amounts of DMSO were added to the

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