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Original Contribution

Gambogic acid induces apoptosis in hepatocellular carcinoma SMMC-7721 cells by targeting cytosolic thioredoxin reductase

Dongzhu Duan, Baoxin Zhang, Juan Yao, Yaping Liu, Jinyu Sun, Chunpo Ge, Shoujiao Peng, Jianguo Fang*

State Key Laboratory of Applied Organic Chemistry and College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou, Gansu 730000, China

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ABSTRACT

The thioredoxin reductase (TrxR) isoenzymes, TrxR1 in cytosol or nucleus and TrxR2 in mitochondria, are essential mammalian selenocysteine (Sec)-containing flavoenzymes with a unique C-terminal -Gly-Cys-Sec-Gly active site. TrxRs are often overexpressed in a number of human tumors, and the reduction of their expression in malignant cells reverses tumor growth, making the enzymes attractive targets for anticancer drug development. Gambogic acid (GA), a natural product that has been used in traditional Chinese medicine for centuries, demonstrates potent anticancer activity in numerous types of human cancer cells and has entered phase II clinical trials. We discovered that GA may interact with TrxR1 to elicit oxidative stress and eventually induce apoptosis in human hepatocellular carcinoma SMMC-7721 cells. GA primarily targets the Sec residue in the antioxidant enzyme TrxR1 to inhibit its Trx-reduction activity, leading to accumulation of reactive oxygen species and collapse of the intracellular redox balance. Importantly, overexpression of functional TrxR1 in cells attenuates the cytotoxicity of GA, whereas knockdown of TrxR1 sensitizes cells to GA. Targeting of TrxR1 by GA thus discloses a previously unrecognized mechanism underlying the biological action of GA and provides useful information for further development of GA as a potential agent in the treatment of cancer.

Natural products and their derivatives are invaluable sources of therapeutic agents and have served humankind in the treatment of various ailments for centuries [1,2]. In recent years, they have also become important molecular probes for studying various cellular processes by virtue of their ability to bind to specific protein targets and interfere with their cellular functions. It is estimated that about half of currently marketed drugs originate from natural products. In the case of anticancer and anti-infective agents, this proportion is even higher [3]. Thus, the past decades have witnessed continuous endeavors to identify the active chemical entities and their molecular targets, leading to the discovery of diverse new therapeutic agents, such as vinblastine, rapamycin, paclitaxel, roscovitin, camptothecin, and homoharringtonine.

Gambogic acid (GA), a major active component of gamboges resin secreted from the *Garcinia hanburryi* tree in southeast Asia, has been used in traditional Chinese medicine for treatment of cancers. Since the initial discovery of GA as an effective apoptosis inducer through a high-throughput screening assay [4], GA has demonstrated potent anticancer activity in numerous types of human cancer cells and has entered phase II clinical trials [5]. A variety of cellular targets have been reported to be affected by GA, including the transferrin receptor [6], NF-κB [7], integrin 1 [8], the 90-kDa heat shock protein [9,10], AMP-activated protein kinase [11], B cell lymphoma 2 [12], thioredoxins [13], p53 [14], topoisomerase [15], and sirtuin 1 [16]. Despite its undoubted anticancer efficacy, the molecular mechanism underlying the action of GA is still elusive, and the primary cellular target and mode of action of this compound remain unclear.

Thioredoxin reductase (TrxR), thioredoxin (Trx), and NADPH comprise a highly conserved, ubiquitous system (the thioredoxin system), which plays a crucial role in maintaining intracellular redox homeostasis [17–22]. Two major isoforms of TrxR/Trx are present in different intracellular organelles: TrxR1/Trx1 are predominantly in the cytosol and nucleus, whereas TrxR2/Trx2 are mainly localized within mitochondria [23]. Despite the different localizations of the isoforms within cells, mammalian TrxR1 and TrxR2 have similar structures and share the same catalytic mechanism. TrxRs catalyze the NADPH-dependent reduction of the disulfide in Trxs, which serve a wide range of functions in cellular redox signaling [18,20,22]. Mammalian TrxRs, compared to those from bacteria, are large selenium-containing proteins [24–26]. Owing to the high reactivity of selenide that is present within the C-terminus of reduced TrxRs, the enzymes have a broad substrate specificity and are easily inactivated by various

* Corresponding author. Fax: +86 931 8915557.
E-mail address: fangjg@lzu.edu.cn (J. Fang).

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alkylating reagents [17,19]. TrxR1 is often overexpressed in many cancer cells and targeting its ablation leads to a reversal in the growth of numerous malignant tumors [27], making this selenoenzyme a promising target for development of novel anticancer agents [17,28–30].

In this study, we disclose that GA displays the most potent cytotoxicity toward human hepatocellular carcinoma SMMC-7721 cells among a panel of cell lines. GA interacts with TrxR1 by primarily targeting the selenocysteine (Sec) residue in the antioxidant enzyme, leading to inhibition of the physiological function of TrxR, accumulation of reactive oxygen species (ROS), and collapse of the intracellular redox balance. As a consequence, GA elicits oxidative stress and eventually induces apoptosis in SMMC-7721 cells. Importantly, overexpression of functional TrxR1 in cells alleviates the cytotoxicity of GA, whereas knockdown of TrxR1 augments the cytotoxicity, supporting the physiological significance of targeting of TrxR by GA.

Materials and methods

Chemicals and enzymes

Recombinant rat TrxR1 (WT TrxR1) was essentially prepared as described [31] and was a gift from Professor Arne Holmgren at Karolinska Institute, Sweden. The recombinant U498C TrxR1 mutant (Sec→Cys) was produced as described [24]. 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 TrxR1 by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) assay. *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 and oxidized glutathione (GSH and GSSG), dimethyl sulfoxide (DMSO), yeast glutathione reductase (GR), Hoechst 33342, puromycin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (Chaps), and 2′,7′-dichlorfluorescein diacetate (DCFH-DA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). NADPH was obtained from Roche (Mannheim, Germany). Fetal bovine serum (FBS) was obtained from Sijiqing (Hangzhou, China). Anti-TrxR1 antibody and dihydroethidium (DHE) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, and streptomycin were obtained from Amresco (Solon, OH, USA). Bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), and anti-actin antibody were obtained from Beyotime (Nantong, China). DTNB was obtained from J&K Scientific (Beijing, China). Short hairpin RNA (shRNA) plasmids targeting TrxR1 (shTrxR1) and nontargeting control (shNT) were a gift from Professor Constantinos Koumenis from the University of Pennsylvania School of Medicine. The preparation of HeLa-shNT and HeLa-shTrxR1 cells was described elsewhere [32]. GA was obtained from Pufei De Biotechnology (Chengdu, China). Iodoacetic acid-conjugated rhodamine B (IAR; Supplementary Fig. S1) was synthesized according to the published procedures [33], and the synthetic route is illustrated in the supplementary material (Supplementary Fig. S1). A 50 mM solution of GA was prepared in DMSO and stored at −20 °C, and the final concentrations of DMSO were no more than 0.1% (v/v) in experiments unless otherwise noted. All other reagents were of analytical grade.

Cell cultures

A549, HeLa, HepG2, SMMC-7721, HEK 293T, and L02 cells, obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and 100 units/ml penicillin/streptomycin and maintained in an atmosphere of 5% CO_2 at 37 °C. HEK cells stably overexpressing TrxR1 (HEK-TrxR1) and those stably transfected with a vector (HEK-IRES) [34,35], kindly provided by Professor Constantinos Koumenis from the University of Pennsylvania School of Medicine, 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 G418 and kept in an atmosphere of 5% CO_2 at 37 °C.

Cytotoxicity assay

The cell viability was measured using the MTT assay. Unless otherwise noted, 5×10^3 cells were incubated with GA or other agents in triplicate in a 96-well plate for the 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% isobutanol, 0.1% HCl) was added, and the cells were incubated overnight at 37 °C. The absorbance was measured at 570 nm using a microplate reader (Thermo Scientific Multiskan GO, Finland).

Generation of stable TrxR1 knockdown cells

The shRNA plasmid targeting coding regions of the TrxR1 gene (shTrxR1) and the control nontargeting shRNA (shNT) were kindly provided by Professor Constantinos Koumenis from the University of Pennsylvania School of Medicine [34]. HeLa cells were plated in a six-well plate with 3×10^5 cells/well in DMEM without antibiotics overnight and were transfected with either shTrxR1 or shNT plasmid using the Gene III Efficiency transfection reagent (Biomiga, San Diego, CA, USA). After 48 h of transfection, the cells were kept in DMEM, 10% FBS, 2 mM glutamine, 100 units/ml penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and selected with 1 μg/ml puromycin.

In vitro TrxR activity assays

DTNB assay [36,37]

The TrxR activity was determined at room temperature using a microplate reader. The NADPH-reduced TrxR (170 nM) or U498C TrxR (700 nM) was incubated with various concentrations of GA for the indicated times at room temperature (the final volume of the mixture was 50 μl) in a 96-well plate. A master mixture in TE buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 μl) containing DTNB and NADPH was added (final concentration: 2 mM and 200 μM, respectively), and the linear increase in absorbance at 412 nm during the initial 3 min was recorded. The same amounts of DMSO were added to the control experiments and the activity was expressed as the percentage of the control.

Endpoint insulin reduction assay [36,37]

The NADPH-reduced TrxR (170 nM) was incubated with various concentrations of GA for 2 h at room temperature in a final volume of 50 μl. A master mixture in TE buffer (50 μl) containing 4 μM *E. coli* Trx, 0.4 mM NADPH, and 0.32 mM insulin was added to the solution, and the incubation continued at room temperature for 0.5 h. Reaction was terminated by the addition of 100 μl 1 mM DTNB in 6 M guanidine hydrochloride (pH 8.0) and the absorbance at 412 nm was measured using a microplate reader. The same amounts of DMSO were added to the control experiments and the activity was expressed as the percentage of the control.