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Original Contribution

Sulfiredoxin inhibitor induces preferential death of cancer cells through reactive oxygen species-mediated mitochondrial damage

Hojin Kim [a,1], Gong-Rak Lee [a,1], Jiwon Kim [a], Jin Young Baek [d], You-Jin Jo [a], Seong-Eun Hong [a], Sung Hoon Kim [a], Jiae Lee [a], Hye In Lee [a], Song-Kyu Park [b], Hwan Mook Kim [c], Hwa Jeong Lee [d], Tong-Shin Chang [d], Sue Goo Rhee [e], Ju-Seog Lee [f], Woojin Jeong [a,*]

[a] Department of Life Science and the Research Center for Cellular Homeostasis, Ewha Womans University, Seoul 120-750, South Korea
[b] College of Pharmacy, Korea University, Sejong 339-700, South Korea
[c] College of Pharmacy, Gachon University of Medicine and Science, Incheon 406-799, South Korea
[d] College of Pharmacy, Ewha Womans University, Seoul 120-750, South Korea
[e] Yonsei Biomedical Research Institute, Yonsei University College of Medicine, Seoul 120-752, South Korea
[f] Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77054, USA

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ABSTRACT

Recent studies have shown that many types of cancer cells have increased levels of reactive oxygen species (ROS) and enhance antioxidant capacity as an adaptation to intrinsic oxidative stress, suggesting that cancer cells are more vulnerable to oxidative insults and are more dependent on antioxidant systems compared with normal cells. Thus, disruption of redox homeostasis caused by a decline in antioxidant capacity may provide a method for the selective death of cancer cells. Here we show that ROS-mediated selective death of tumor cells can be caused by inhibiting sulfiredoxin (Srx), which reduces hyperoxidized peroxiredoxins, leading to their reactivation. Srx inhibitor increased the accumulation of sulfinic peroxiredoxins and ROS, which led to oxidative mitochondrial damage and caspase activation, resulting in the death of A549 human lung adenocarcinoma cells. Srx depletion also inhibited the growth of A549 cells like Srx inhibition, and the cytotoxic effects of Srx inhibitor were considerably reversed by Srx overexpression or antioxidants such as N-acetyl cysteine and butylated hydroxyanisol. Moreover, Srx inhibitor rendered tumorigenic ovarian cells more susceptible to ROS-mediated death compared with nontumorigenic cells and significantly suppressed the growth of A549 xenografts without acute toxicity. Our results suggest that Srx might serve as a novel therapeutic target for cancer treatment based on ROS-mediated cell death.

1. Introduction

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), have been known to be generated as by-products in aerobic metabolism or intentionally produced as an intracellular messenger in response to a variety of extracellular stimuli [1–3]. The level of ROS is critical since a moderate level of ROS can promote cell proliferation and differentiation while excessive amounts of ROS can be toxic to the cells [4]. When cellular production of ROS overwhelms cellular antioxidant capacity, the ROS are able to inflict serious cellular damage and they have been implicated in the pathogenesis of cancer, cardiovascular, inflammatory, and degenerative diseases [2,3,5]. Thus, maintaining redox balance is crucial for cellular homeostasis.

Many reports have shown not only increased generation of ROS [6,7], but also elevated expression of antioxidant enzymes in a wide range of cancer cells [8]. Due to the toxicity of excessive levels of ROS to cells, cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents. Therefore, disruption of redox homeostasis due to a decline in antioxidant capacity is a possible solution to selectively kill cancer cells without causing significant

Abbreviations: BHA, butylated hydroxyanisol; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; H_2O_2, hydrogen peroxide; $\Delta\Psi_m$, mitochondrial membrane potential; MPTP, mitochondrial permeability transition pore; NAC, N-acetyl cysteine; NAO, nonyl acridine orange; PO-1, Peroxy Orange-1; Prxs, peroxiredoxins; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; SD, standard deviation; Srx, sulfiredoxin; Trx, thioredoxin

* Correspondence to: Science Building C, Room 211, 11-1 Daehyun-dong, Seodaemun-gu, Seoul 120-750, South Korea.
E-mail address: jeongw@ewha.ac.kr (W. Jeong).

[1] These authors equally contributed to this work.

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toxicity to normal cells [9].

Peroxiredoxins (Prxs), a family of peroxidases with the ability to reduce H_2O_2 and alkyl hydroperoxides, have been known to be involved in many cellular functions including proliferation, cell cycle, apoptosis, and differentiation as well as the cellular protection against oxidative stress [8,10,11]. There are six mammalian Prx isoforms that are distributed in most cellular compartments including cytosol, mitochondria, nucleus, endoplasmic reticulum, and peroxisomes, and these isoforms are classified into three subfamilies: four typical 2-Cys Prxs (Prx I-IV), one atypical 2-Cys Prx (Prx V), and one 1-Cys Prx (Prx VI) [10]. Members of the typical 2-Cys Prxs are inactivated via hyperoxidation of the active site cysteine to sulfinic acid during catalysis [12] and are reactivated via an ATP-consuming reaction catalyzed by sulfiredoxin (Srx) [13–16].

Srx catalyzes the transfer of the γ-phosphate of ATP to the sulfinic cysteine of Prx and the resulting sulfinic phosphoryl ester is reduced by thiol equivalents such as thioredoxin (Trx) and glutathione [16–18]. In addition to its sulfinic acid reductase activity, Srx possesses deglutathionylation activity [19,20]. Recent findings have shown that Srx is induced by a variety of stimuli or chemicals that result in oxidative damage in Srx-deficient cells [21,22]. It was reported that an enhanced level of Srx promotes cell proliferation [23], and Srx is highly expressed in several human tumors including skin tumors, rectal carcinoma and lung adenocarcinoma, compared with the levels found in corresponding normal tissues [24,25]. Srx was also found to be required for anchorage-independent colony formation by human lung cancer cells and for cell migration and invasion in vitro, as well as for the formation of metastases by these cells in vivo [26].

Srx seems to be important for cellular redox homeostasis and cancer progression. Additionally, induction of Srx under a variety of conditions and elevated expression of Srx in cancer cells are likely to be adaptive responses to oxidative stress, used to promote cell survival. We recently developed a colorimetric assay method for high-throughput screening of Srx inhibitors and isolated two compounds, D1 and E7, through screening of 25,000 chemicals for Srx inhibition [27]. In this study, we show that Srx inhibitor could selectively kill cancer cells through an ROS-mediated mechanism. Our results suggest that Srx might be a potential therapeutic target for cancer treatment.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human Prx I, Trx, and Srx were prepared as described previously [14]. D1 and its derivative chemicals were purchased from AMRI. Dithiothreitol, glutathione, dimethyl sulfoxide (DMSO), ATP, N-acetyl cysteine (NAC), and butylated hydroxyanisol (BHA) were purchased from Sigma-Aldrich. The fluorogenic caspase substrates DEVD-AMC and LEHD-AMC were purchased from BD Pharmingen and Biomol, respectively. Rabbit polyclonal antibodies specific for PrxI and sulfinic Prx (Prx-SO_2) were purchased from AbClone. Rabbit polyclonal antibodies specific for β-actin and cytochrome C were purchased from Abcam and Santa Cruz Biotechnology, respectively.

2.2. Cell culture and transfection

A549 human lung adenocarcinoma cells were cultured in Ham's F-12K medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were transfected transiently using Fugene HD reagent according to the manufacturer's instructions (Roche Applied Science). T80 or T80H Ras-transformed human ovarian cancer cells were cultured in MCDB 105/M199 (1:1) medium supplemented with 10 ng/ml epidermal growth factor, 10% fetal bovine serum, and 1% penicillin–streptomycin [28].

2.3. Measurement of cell viability

Cells were plated in 96-well plates. The 10 μl of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent (Roche Applied Science) was added into each well. After incubation at 37 °C for 2–4 h, the absorbance at 450 nm was measured.

2.4. Colony forming assay

Cells were seeded at a density of 1×10^4 cells on a 6-well plate and were grown for 8 days. The cells were washed with phosphate-buffered saline (PBS) and fixed by 2% paraformaldehyde at 25 °C for 10 min. The cells were stained with 1% crystal violet (Sigma Aldrich) in 100% ethanol for 1 h at 25 °C and were photographed. After solubilizing the stain in 100% ethanol, the colony forming ability was quantified by measuring the absorbance at 590 nm.

2.5. Determination of Srx activity in cells

Cells were exposed to 200 μM H_2O_2 for 10 min. After removal of H_2O_2, the cells were incubated in fresh medium, washed twice with PBS, and lysed in lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM 4-(2-Aminoethyl) benzene sulfonyl fluoride, 10 μg/ml apoprotinin, and 10 μg/ml leupeptin]. The lysate (10 μg) was subjected to immunoblot analysis with antibodies against sulfinic PrxI and PrxI.

2.6. Flow cytometry analysis

For measurement of intracellular ROS or H_2O_2, cells were incubated in the dark for 30 min with 5 μM 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes) or 5 μM Peroxy Orange-1 (PO-1) (Tocris Bioscience) for 15 min in phenol red-free medium, respectively [29,30]. For analysis of cell death, cells were labeled with fluorescein isothiocyanate (FITC)-Annexin V plus propidium iodide (PI) in annexin binding buffer at 25 °C for 15 min, according to the manufacturer's instructions (FITC Annexin V apoptosis detection kit; BD Biosciences). For measurement of mitochondrial membrane damages, cells were stained with either 50 nM nonyl acridine orange (NAO) or 50 nM tetramethylrhodamine ethyl ester (TMRE) at 37 °C for 30 min. The cells were washed with PBS and analyzed immediately using a FACS Calibur flow cytometer (BD Biosciences). The excitation wavelength was 488 nm, and the observation wavelength was 530 nm for green fluorescence and was 585 nm for red fluorescence. Relative change in fluorescence was analyzed with WinMDI software.

2.7. Mitochondria cytochrome c release

Cells were harvested and resuspended in permeabilization buffer (200 μg/ml digitonin and 80 mM KCl in PBS). After 5 min of incubation on ice, samples were centrifuged at 1000g for 5 min. The supernatant (cytosolic fraction) was immediately transferred to a new tube and the pellet (mitochondria fraction) was resuspended in RIPA buffer. Both fractions were then subjected to immunoblot analysis for cytochrome c.