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Original Contribution

Salvicine triggers DNA double-strand breaks and apoptosis by GSH-depletion-driven H_2O_2 generation and topoisomerase II inhibition

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ABSTRACT

Glutathione (GSH), as the major small-molecule antioxidant in cells, has been implicated in the regulation of cell proliferation and apoptosis. Salvicine (SAL), a novel diterpenoid quinone compound, exhibits potent antitumor activities both in vitro and in vivo by poisoning topoisomerase II (Topo II) and has entered Phase II clinical trials for cancer therapy. Herein, we provide further evidence that SAL-induced DNA double-strand breaks (DSBs) and apoptosis by GSH depletion drives H_2O_2 generation and Topo II inhibition. Our data reveal that treatment with SAL results in a pronounced increase in intracellular H_2O_2 and is accompanied by the occurrence of DNA DSBs and apoptosis in epithelial HeLa cells. Furthermore, SAL was also noted to trigger a dramatic depletion of intracellular GSH via its direct reaction with GSH. Importantly, the introduction of GSH and overexpression of catalase antagonized SAL-mediated DNA DSBs and apoptosis, and the GSH synthesis inhibitor dl-buthionine-[*S,R*]-sulfoximine reduced SAL-mediated H_2O_2 generation, indicating that SAL-mediated H_2O_2 generation is derived from intracellular GSH depletion. Notably, SAL-mediated Topo II inhibition was also concentration-dependently reversed by GSH. Furthermore, we found that Topo II-defective HL-60/MX2 cells were almost completely resistant to SAL-induced DNA DSBs, suggesting that, in addition to its direct inhibitory effect on Topo II, SAL-mediated H_2O_2 generation may also trigger DNA DSBs via poisoning of Topo II. All these findings together suggest that GSH-depletion-driven H_2O_2 generation and Topo II inhibition are both critical for SAL-induced DNA DSBs and apoptosis.

Reactive oxygen species (ROS) are involved in a variety of physiological and pathological processes. Low levels of ROS regulate cellular signal transduction and play important roles in normal cell proliferation [1]. High levels of ROS lead to apoptosis and necrosis, which are implicated in cancer, aging, and neurodegenerative disorders [2,3]. Increasing studies have indicated that ROS are involved in anticancer drug-mediated apoptosis [4,5]. Because ROS are chemically reactive and potentially toxic to cells when they accumulate to high levels, most cells have an elaborate and highly regulated defense system to promptly minimize adverse cellular effects resulting from excessive exposure to ROS. This includes a broad spectrum of metabolic enzymes and small-molecule antioxidant defense systems such as superoxide dismutase (SOD), catalase, and the glutathione (GSH) redox system. Of them, GSH stands out as a main and integral oxidant scavenger, which reacts as either a one-electron donor to radicals or a two-electron donor to electrophiles and, in particular, occurs in all mammalian cell types. In fact, GSH redox status has been recognized to be critical for various biological events, including transcription activation of specific genes, regulation of redox-related signal transduction pathways, and control of cell proliferation and apoptosis [6,7]. Encouragingly, recent evidence has further emphasized the significance of intracellular GSH in the anticancer therapy of some antitumor drugs, simply because the cytotoxicity of antitumor drugs may largely depend on intracellular levels of GSH, and GSH depletion may facilitate ROS accumulation and potentiate the lethality in antitumor drug-treated cells [8–10].

Salvicine (SAL) is a novel diterpenoid quinone compound synthesized by the structural modification of a natural product isolated from the Chinese medicinal herb *Salvia prionitis lance* [11]. SAL possesses potent in vitro and in vivo activities against malignant tumor cells, especially in some human solid tumor models, and has now entered Phase II clinical trials [12,13]. SAL induces apoptosis in various human tumor cell lines and displays prominent activity against multiple-drug resistance [14–18]. Mechanistic studies have shown that Topo II functions as one of the primary molecular targets of SAL [19,20]. Our

Abbreviations: ATZ, 3-amino-1,2,4-triazole; BSO, dl-buthionine-[*S,R*]-sulfoximine; CAT, catalase; DCF, 2,7-dichlorofluorescein; $DCFH_2$-DA, 2,7-dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium chloride; DSB, double-strand break; GSH, glutathione; HE, hydroethidine; kDNA, kinetoplast DNA; MTT, tetrazolium bromide; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; SAL, salvicine; SOD, superoxide dismutase; SRB, sulforhodamin B; Topo II, topoisomerase II; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; VP-16, etoposide.

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recent study further revealed that SAL is also capable of triggering ROS generation, which was suggested to contribute only partially to the apoptosis of MCF-7 cells [21].

Because the generation of ROS has been recognized to mediate reversible Topo II cleavable complex formation by covalently modifying Topo II, and in particular some quinone compounds have been demonstrated to modify the thiol groups of Topo II cysteine residues and lead to the reversible cleavable complex formation [22,23], we are thus encouraged to address whether SAL is able to offer a bridge between ROS and Topo II cleavable complex formation via the quinone-based settings. Moreover, intracellular GSH levels have been increasingly accepted as responding to the critical accumulation of ROS that are responsible for apoptosis-inducing effects in antitumor drug-treated cells [10]. We are further interested in asking if GSH responds to ROS-driven SAL-mediated DNA DSBs and apoptosis.

The aim of the present study was to unravel the relationship between ROS and Topo II inhibition in SAL-mediated DNA DSBs, to characterize the type(s) of ROS involved in SAL-mediated apoptosis, and to explore the possible role of GSH in SAL-driven biological events, in the hope of furthering our understanding of the distinct or unidentified roles of SAL in cancer therapy.

Material and methods

Cell lines

HeLa, HL-60, and Topo II-deficient HL-60/MX2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HL-60/MX2 cells, a mitoxantrone-resistant variant of HL-60, are known to be resistant to Topo II poisons because of a lowered Topo IIα content and absence of Topo IIβ [24]. HeLa, HL-60, and HL-60/MX2 cells were maintained in MEM (HeLa cells; GIBCO, Grand Island, NY, USA) and RPMI 1640 medium (HL-60 and HL-60/MX2; GIBCO), supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a 5% CO_2 humidified environment.

Drugs and reagents

SAL was kindly provided by Professor Jin-Sheng Zhang from the Department of Phytochemistry, Shanghai Institute of Materia Medica. Its purity was greater than 99.8%, as determined by HPLC [11]. The compound was solubilized to 40 mM in dimethyl sulfoxide as a stock solution and stored at −20°C in the dark. Aliquots were thawed just before each experiment and diluted to the indicated concentrations with normal saline. Purified human DNA topoisomerase II (hTopo II) α and kinetoplast DNA (kDNA) were purchased from TopoGEN, Inc. (Columbus, OH, USA). Tetrazolium bromide (MTT), sulforhodamin B (SRB), GSH, *N*-acetylcysteine (NAC), SOD, catalase (CAT), dl-buthionine-[*S*,*R*]-sulfoximine (BSO), diphenyleneiodonium chloride (DPI), Trolox, 2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), hydroethidine (HE), etoposide, camptothecin (CPT), and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3-Amino-1,2,4-triazole (ATZ) and hydrogen peroxide (H_2O_2) were purchased from Fluka (Buchs SG, Switzerland).

H_2O_2 and $O_2^{\cdot-}$ measurement

Accumulation of intracellular H_2O_2 and $O_2^{\cdot-}$ was detected with the probes $DCFH_2$-DA and HE, respectively, as described previously [25,26]. In brief, after treatment, cells were labeled with 5 μM HE or 10 μM $DCFH_2$-DA for 20 min at 37°C in a humidified atmosphere at 5% CO_2. The labeled cells were washed and immediately fixed with 4% paraformaldehyde in the dark for 20 min at 4°C and coated with one drop of mounting medium (DakoCytomation, Carpinteria, CA, USA). Images were photographed using a Leica TCS confocal microscope (Leica, Deerfield, IL, USA). To quantify H_2O_2 and $O_2^{\cdot-}$, the fluorescence intensity (HE, FL-2 channel; $DCFH_2$-DA, FL-1 channel) was measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

Plasmid construction and transfection

The pCAT10 plasmid containing the human catalase cDNA was obtained from the ATCC. The pcDNA.3.1/V5-His A, B, and C mammalian expression vector was purchased from Invitrogen Corp. (San Diego, CA, USA). A 1.7-kb fragment containing human catalase cDNA from pCAT10 was amplified by PCR using primers with *Kpn*I/*Not*I ends (5′-ATATGGT-ACCGCCTGCTGAGGGTGGAGA-3′ and 3′-AAGTAGGCACATTGGG-CGCGCCGGCGATAAT-5′), and the resulting PCR product was inserted into the *Kpn*I/*Not*I sites of pcDNA.3.1/V5-His A, B, and C to obtain the final recombinant pcDNA.3.1-CAT [27]. Each DNA insert was sequenced bidirectionally by using the Taq DyeDeoxy terminator cycle sequencing kit and an ABI Model 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). HeLa cells were seeded onto six-well plates and grown to 95% confluence. The plasmid pcDNA.3.1-CAT and the empty vector were transfected into HeLa cells using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the instructions provided by the manufacturer. After transfection for 2 days, catalase protein expression and activity were measured.

RT-PCR analysis

Total RNA in HeLa cells transfected with pcDNA3.1-CAT or empty vector was extracted using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RNA concentrations were measured by a spectrophotometric method. Total RNA (2 μg) was subjected to reverse transcription and PCR amplification using a Qiagen OneStep RT-PCR kit. The following primers were used: 5′-ATATGGTACCGCCTGCTGAGGGTGGAGA-3′ (sense) and 3′-AAGTAGGCACATTGGGCGCGCCGGCGATAAT-5′ (antisense) for CAT and 5′-CCATGGAGAAGGCTGGGG-3′ (sense) and 3′-CCAGTAGGTACTGTTGAAAC-5′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase. The amplified products were separated on 1% agarose gel, stained with ethidium bromide (EBr), and photographed using a Syngene gel analysis system.

Catalase activity determination

The catalase activity was measured using a commercial catalase assay kit (Cayman Chemical, Ann Arbor, MI, USA). Briefly, after treatment, cells were washed, scraped, and centrifuged at 2000 *g* for 10 min at 4°C. The cell pellets were resuspended in 25 mM potassium phosphate (pH 7.5), 1 mM EDTA, and 0.1% BSA and pulse-sonicated on ice using an ultrasonic processor (Sonics and Materials, Inc., Newtown, CT, USA) and centrifuged at 10,000 *g* for 15 min at 4°C. The catalase activity in the supernatant was measured according to the reaction of catalase with methanol that gives rise to formaldehyde in the presence of an optimal concentration of H_2O_2. The optical density of formaldehyde at 540 nm was read using a VERSAmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The catalase activity was calculated using the following equations: formaldehyde (μM) = 8.5 × (sample absorbance − *y*-intercept/slope) and catalase activity = μM formaldehyde/20 min × sample dilution.

GSH measurement

The GSH in cells was measured using a GSH quantification kit (Dojindo Molecular Technologies, Gaithersburg, MD, USA) [28]. Briefly, after treatment, cells were scraped and centrifuged at 1000 *g* for 10 min at 4°C, and the cell pellets were resuspended, lysed, and centrifuged at 8000 *g* for 10 min at 4°C according to the manufacturer's instructions. The GSH was measured by the glutathione reductase recycle assay. The