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Auranofin, an inhibitor of thioredoxin reductase, induces apoptosis in hepatocellular carcinoma Hep3B cells by generation of reactive oxygen species

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Abstract. Mammalian thioredoxin reductase (TrxR) plays a vital role in restoring cellular redox balance disrupted by reactive oxygen species (ROS) generation and oxidative damage. Here, we evaluated whether auranofin, a selective inhibitor of TrxR, could serve as a potential anti-cancer agent through its selective targeting of TrxR activity in Hep3B hepatocellular carcinoma cells. Auranofin treatment reduced the TrxR activity of these cells and induced apoptosis, which were accompanied by up-regulation of death receptors (DRs) and activation of caspases, as well as promotion of proteolytic degradation of poly(ADP-ribose)-polymerase. Treatment with a pan-caspase inhibitor reversed the auranofin-induced apoptosis and growth suppression, indicating that auranofin may induce apoptosis through a caspase-dependent mechanism involving both the intrinsic and extrinsic apoptotic pathways. Auranofin also significantly altered mitochondrial function, promoting mitochondrial membrane permeabilization and cytochrome *c* release by regulating Bcl-2 family proteins; these events were accompanied by an accumulation of ROS. Inhibition of ROS generation with the ROS quencher significantly attenuated the inactivation of TrxR in auranofin-treated cells and almost completely suppressed the auranofin-induced up-regulation of DRs and activation of caspases, thereby preventing auranofin-induced apoptosis and loss of cell viability. Taken together, these findings indicate that auranofin inhibition of TrxR activity in Hep3B cells activates ROS- and caspase-dependent apoptotic signaling pathways and triggers cancer cell death.

Key words: TrxR — Auranofin — Apoptosis — Caspase — ROS

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Introduction

Reactive oxygen species (ROS) are chemically reactive molecules that form as common byproducts of normal aerobic cellular metabolism (Bae et al. 2011; Woolley et al. 2013). ROS play an essential role in the regulation of the normal physiology of a cell, but high levels of ROS can cause oxidative stress that can lead to cell death by triggering apoptotic pathways (Matés and Sánchez-Jiménez 2000; Wu 2006). This oxidative stress-induced cell death occurs due to ROS-induced depolarization of the mitochondrial membrane, which promotes the release of apoptotic factors from the mitochondria to the cytosol (Simon et al. 2000; Paradies et al. 2002). These apoptotic factors then trigger apoptosis, or programmed cell death, which maintains tissue homeostasis and facilitates the removal of damaged cells (Evans 1993; Hengartner 2000).

Apoptosis involves a series of biochemical pathways, or signaling cascades that can be activated by either intracellular or extracellular events to commit a cell to undergo apoptosis (Hengartner 2000; Fulda and Debatin 2004). In mammals, these apoptotic signaling cascades can be divided into two broad categories: the extrinsic pathway cascade that involves receptor-mediated interactions and the intrinsic pathway cascade mediated by mitochondrial stimuli (Hale et al. 1996; Debatin and Krammer 2004). Some crosstalk occurs between the two pathways and both require the activation of caspases, a family of cysteine proteases that function as the executors of apoptosis (Fulda and Debatin 2004; Gloire et al. 2008).

The apoptotic process can be disrupted by numerous cellular signaling pathways involved in cell survival and proliferation. One of these is the thioredoxin (Trx) system, a thiol-dependent electron donor system comprising Trx, Trx reductase (TrxR), and nicotinamide adenine dinucleotide phosphate (NADPH) (Kondo et al. 2006; Holmgren and Lu 2010). TrxR is a NADPH-dependent seleno cysteine-containing flavoenzyme that catalyzes the reduction of oxidized Trx and other small molecular oxidants (Mustacich and Powis 2000; Arner 2009). The Trx system therefore plays critical roles in maintaining cellular redox homeostasis by counteracting the effects of ROS and regulating redox-related signaling cascades (Arner and Holmgren 2000; Nguyen et al. 2006). However, the strong antioxidant activity of the Trx system also counteracts apoptotic signals, allowing the uncontrolled cell proliferation and tumor growth that are the hallmarks of cancer (Mustacich and Powis 2000; Go and Jones 2013). Indeed, accumulating evidence now indicates that Trx/TrxR is overexpressed or constitutively active in many human primary cancers, and this activity supports cancer development by promoting cell growth, invasion, and metastasis; inhibiting of the normal apoptotic mechanisms; and inducing resistance to chemotherapy agents (Holmgren and Lu 2010; Liu et al. 2012).

Interestingly, TrxR knockdown by stable transfection with a small interfering RNA construct has been shown to nearly abolish the capacity of lung cancer cells to form tumors in a xenograft model (Yoo et al. 2006). Similarly, overexpression of the alternative splicing variant of TrxR results in a marked induction of apoptosis in cervical cancer cells (Chang et al. 2005). These experiments indicate an essential role of TrxR in cancer cell growth; therefore, the development of drugs that inhibit TrxR activity may represent a key strategy for the treatment and prevention of cancer (Powis and Kirkpatrick 2007; Mahmood et al. 2013).

One well-known inhibitor of TrxR is auranofin, a gold-containing triethylphosphine compound. Originally approved to treat rheumatoid arthritis, auranofin is now recognized as a potent TrxR inhibitor with novel anticancer activities (Craig et al. 2012; Roder and Thomson 2015). This anticancer activity is apparently independent of tumor resistance to anti-cancer drugs such as imatinib and cisplatin, but is closely related to the endogenous and inducible levels of ROS in the cancer cells (Rigobello et al. 2002; Marzano et al. 2007; Pessetto et al. 2013). Recent studies now indicate that auranofin induces an excessive production of ROS and triggers endoplasmic reticulum stress, thereby leading to cancer cell apoptosis (Liu et al. 2000; Fiskus et al. 2014; Zou et al. 2015). However, the molecular mechanisms underlying the anticancer effects of auranofin are not yet completely understood.

This study examined the pro-apoptotic effects of auranofin in the Hep3B human hepatocellular carcinoma cell line. Treatment with auranofin decreased Hep3B cell viability and TrxR activity, and increased ROS generation when compared to untreated controls. Further evaluation of the importance of ROS as critical mediators of auranofin-induced Hep3B cell death supported a sequence of events leading to the activation of downstream caspases and apoptosis.

Materials and Methods

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin were purchased from WELGENE (Daegu, Republic of Korea). Auranofin was obtained from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemicals Co.); further dilutions were made in DMEM. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC), 4,6-diamidino-2-phenyllindile (DAPI), phenol:chloroform:isoamyl alcohol, RNase A, ethidium

bromide (EtBr), propidium iodide (PI), paraformaldehyde, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) and 2',7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich Chemicals Co. Annexin V-fluorescein isothiocyanate (FITC) and caspase activity assay kits were bought from R&D Systems (Minneapolis, MN, USA). TrxR assay kit and a pan-caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) were bought from Cayman Chemical Co. (Ann Arbor, MI, USA) and Calbiochem (San Diego, CA, USA), respectively. Proteinase K and enhanced chemiluminescence (ECL) kit were purchased from Invitrogen (Carlsbad, CA, USA) and Amersham Co. (Arlington Heights, IL, USA), respectively. A mitochondrial fractionation kit was obtained from Active Motif (Carlsbad, CA, USA). Primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Chemicon (Temecula, CA, USA), and Sigma-Aldrich Chemicals Co. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from Amersham Co. All other chemicals not specifically mentioned here were purchased from Sigma-Aldrich Chemicals Co.

Cell culture and cell viability assay

Hep3B cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in an incubator at 37°C with a 5% CO_2 atmosphere. Measurement of cell viability was determined using the MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. In brief, cells were seeded in 6-well plates at a density of 1×10^5 cells/well, incubated to stabilize for 24 h, and then treated with various concentrations of auranofin for 24 h. Following treatment, the cells were incubated with an MTT solution (0.5 mg/ml) at 37°C in the dark. After 2 h, the medium was removed and the formazan crystals were solubilized in DMSO. The absorbance of each well was measured at 540 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA).

Determination of TrxR activity

Following treatment with the test drug(s), cells were lysed and TrxR activity was determined in the protein lysate using a commercially available kit, following the manufacturer's instructions. Briefly, 40 µl of the reaction mix (30 µl assay buffer, 8 µl 5, 5′-dithiobis (2-nitrobenzoic) acid solution and 2 µl NADPH) was added to each sample and incubated at 25°C. A blank sample, containing everything except Trx, was treated in the same manner. After 20 min incubation, the absorbance at 412 nm was measured with an ELISA reader, and the blank value was subtracted from the corresponding absorbance value of the sample. The same amounts of DMSO were added to the control experiments and the activity was expressed as the percentage of the control.

DNA fragmentation assay

Cells were lysed for 1 h at room temperature in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100. The lysates were vortexed and cleared by centrifuging at 14,000 rpm for 30 min at 4°C, followed by treatment of the supernatants with proteinase K for 3 h at 50°C. The DNA in the supernatant was extracted by addition of an equal volume of neutral phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). Isopropanol and 5 M NaCl were added to the upper aqueous layer and incubated for 6 h at 20°C. After centrifugation for 15 min at 14,000 rpm, the DNA pellets were air dried and dissolved in 20 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA) with 300 µg/ml of RNase. The DNA samples were separated on 1.5% agarose gels containing 0.1 µg/ml EtBr and viewed under an ultraviolet light.

Fluorescence microscopy examination of apoptosis

The cells were treated with auranofin for 24 h, harvested, washed in ice-cold phosphate-buffered saline (PBS), and fixed in 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, and stained with 2.5 µg/ml DAPI solution (excitation wavelength, 340 nm; emission wavelength, 488 nm) for 10 min at room temperature. Changes in the nuclear morphology of the cells were captured using a fluorescence microscope (Carl Zeiss, Jena, Germany) (Park et al. 2014a).

Measurement of apoptosis by flow cytometry analysis

The magnitude of apoptosis was determined using an Annexin-V FITC apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). The cells were washed with PBS and suspended in annexin-V binding buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM $CaCl_2$, according to the manufacturer's protocol. Cell aliquots were incubated with annexin-V FITC, mixed, and incubated for 15 min at room temperature in the dark. PI (5 µg/ml) was then added to stain the necrotic cells. The numbers of apoptotic cells were determined by fluorescence-activated cell sorter analysis in a flow cytometer at an excitation wavelength of 488 nm and emission wavelength of 525 nm for FITC fluorescence and 620 nm for PI fluorescence (Becton Dickinson, San Jose, CA, USA) (Kwon et al. 2015).

Determination of mitochondrial membrane potential (MMP)

The MMP values were determined using the dual-emission potential-sensitive probe, JC-1 (excitation wavelength, 520 nm; emission wavelength, 596 nm), which is internalized and concentrated by respiring mitochondria and can therefore reflect MMP changes in cells. In brief, cells were fixed and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, and then incubated with 10 μM JC-1 for 30 min at 37°C in the dark. Subsequently, the cells were washed with PBS to remove unbound dye, and the amount of JC-1 retained by 10,000 cells *per* sample was measured at 488 and 575 nm using a flow cytometer (Kim et al. 2014).

Measurement of ROS

Generation of intracellular ROS was examined by flow cytometry using DCF-DA (excitation wavelength, 485 nm; emission wavelength, 530 nm). Briefly, cells from each treatment were harvested, washed twice with PBS, and then resuspended in 10 μM DCFH-DA at 37°C for 20 min in a dark room. ROS production in the cells was monitored immediately by a flow cytometry (Seo et al. 2014). The involvement of elevated ROS in auranofin-induced apoptosis was confirmed by preincubating the cells with NAC, an established antioxidant, for 2 h before treatment with auranofin.

Western blot analysis

The cells were lysed in an extraction buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1 mM sodium orthovanadate, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonylfluoride] for 30 min at 4°C. In parallel experiments, the cytosolic and mitochondrial fractions were isolated using a commercial fractionation kit according to the manufacturer's instructions. Equal amounts of protein samples were then separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked for 1 h at room temperature with 5% non-fat dried milk in PBS containing 0.05% Tween-20 and then incubated overnight at 4°C with specific antibodies. Protein bands were observed after treatment with appropriate horseradish peroxidase-conjugated secondary antibodies using an ECL method according to the recommended procedure.

Statistical analysis

The results are presented as the means ± SD for at least three independent experiments performed in triplicate. Data were analyzed for statistical significance through a one-way analysis of variance. A value of $p < 0.05$ was considered statistically significant.

Results

Auranofin suppresses cell viability and inhibits TrxR activity in Hep3B cells

The effect of auranofin on cell viability of Hep3B cells was investigated by exposing the cells to various concentrations of auranofin for 24 h and then measuring cell viability by the MTT assay. As shown in Fig. 1A, auranofin treatment decreased cell viability in a dose-dependent manner. For example, treatment with 3 μM and 4 μM for 24 h resulted in approximately 47% and 75% inhibition, respectively. The

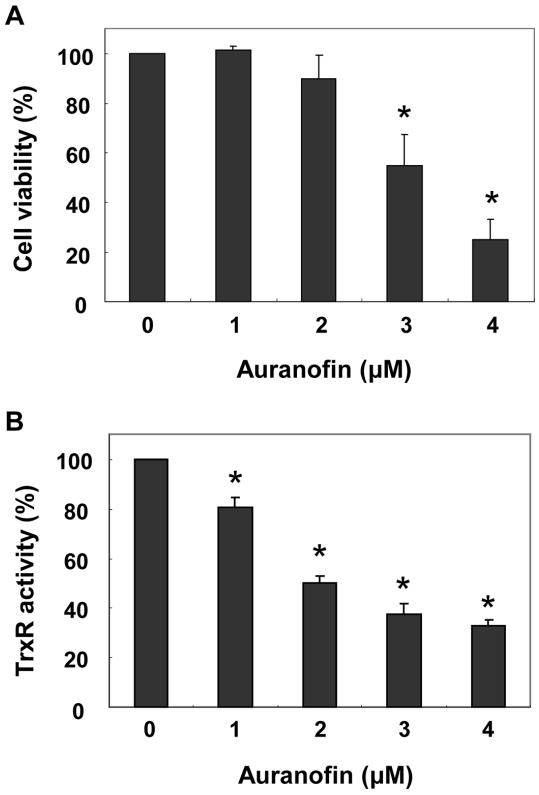

Figure 1. Auranofin suppresses cell viability and inhibits TrxR activity in hepatocellular carcinoma Hep3B cells. **A.** Cells were treated with the indicated concentrations of auranofin. After 24 h incubation, the MTT assay was performed. **B.** The effects of auranofin on TrxR activity in cells grown under the same conditions as (A) were measured using a TrxR colorimetric assay and presented as a percentage of the untreated control. Each point represents the mean ± SD of three independent experiments (* $p < 0.05$ *vs.* control group).

possibility that the inhibitory effects of auranofin on cell viability were associated with the modulation of TrxR activity was then examined. Auranofin treatment concentration-dependently decreased the TrxR activity in Hep3B cells (Fig. 1B), suggesting that the auranofin-induced inhibition of cell viability was associated with a reduction in TrxR activity in Hep3B cells.

Auranofin induces apoptotic cell death in Hep3B cells

DNA fragmentation assays were performed to determine whether the inhibition of cell viability by auranofin in Hep3B cells was caused by the induction of apoptosis. As shown in Fig. 2A, agarose gel electrophoresis indicated an increased and concentration-dependent accumulation of fragmented DNA in Hep3B cells in response to auranofin treatment. DAPI staining revealed an increase in morphological changes, such as nuclear condensation and fragmentation, in cells treated with auranofin, indicating an increasing number of apoptotic cells. No increases were observed in the control cells (Fig. 2B). The rate of apoptotic cell death was then assessed by staining auranofin-treated cells with annexin V-FITC and PI for flow cytometry analysis. Auranofin increased the percentage of annexin V positive Hep3B cells in a dose-dependent manner (Fig. 2C).

Auranofin induces mitochondrion-mediated apoptosis in Hep3B cells

The mechanism of the auranofin-induced apoptosis in Hep3B cells was explored by determining the effect of auranofin on the MMP, a hallmark of intrinsic apoptosis (Breckenridge and Xue 2004). The flow cytometry results demonstrated that auranofin treatment caused a significant and concentration-dependent reduction in the MMP

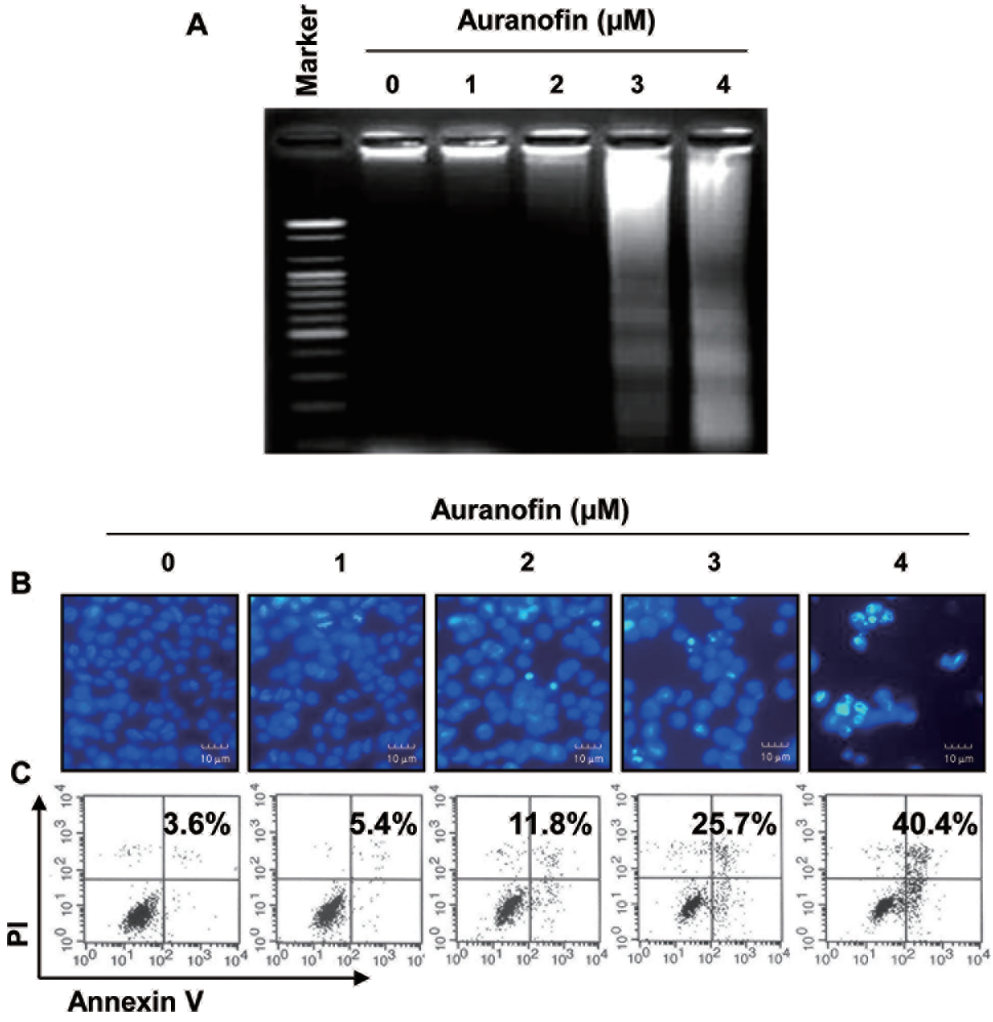

Figure 2. Auranofin induces apoptosis in Hep3B cells. Cells were incubated with the indicated concentrations of auranofin for 24 h. **A.** DNA fragmentation was analyzed by extracting genomic DNA, electrophoresis in a 1.5% agarose gel, and then visualizing by EtBr staining. **B.** The cells were fixed and stained with a DNA-specific fluorescent dye (DAPI) solution. The stained nuclei were observed with a fluorescence microscope (original magnification, 400×; scale 10 μm). **C.** The degree of apoptosis induced by auranofin was determined in cells stained with FITC-conjugated Annexin V and PI for DNA and subjected to flow cytometry analysis. Apoptotic cells are determined by counting the % of annexin V^+/PI^- cells. Each point represents the means of two independent experiments.

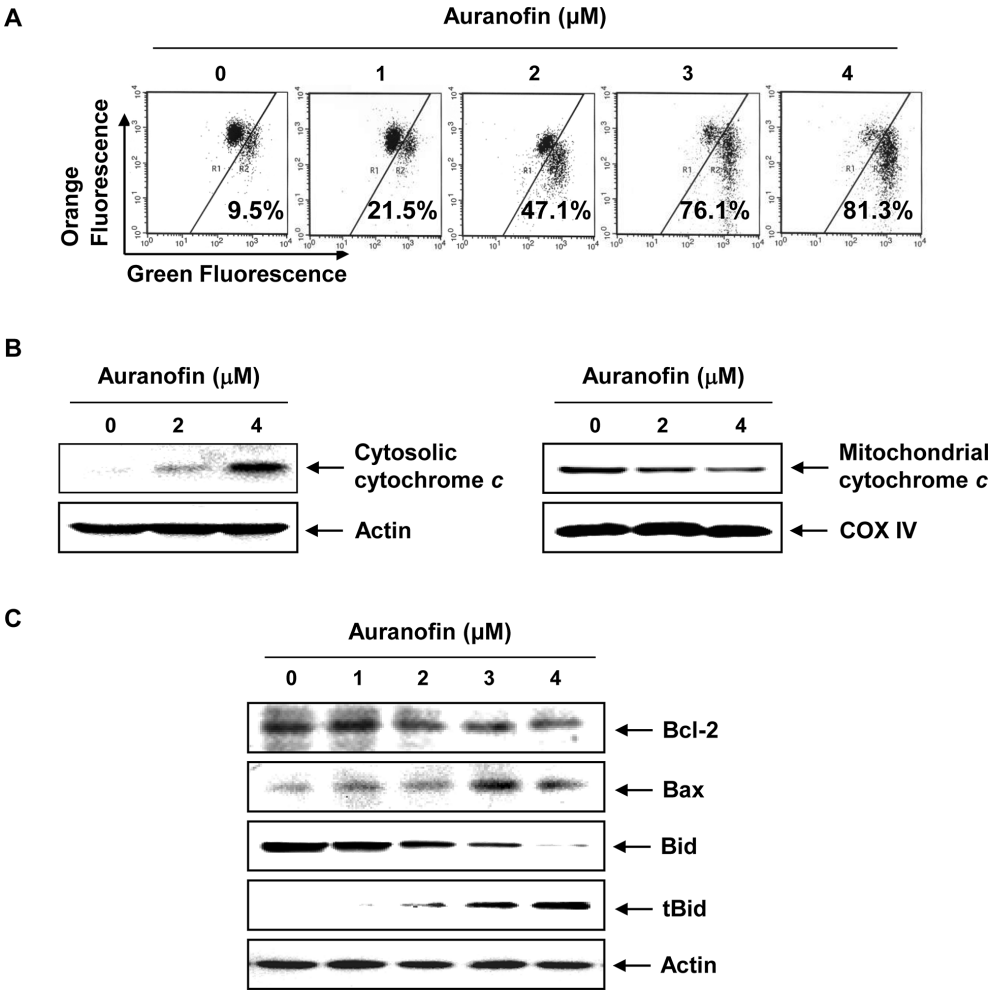

Figure 3. Effects of auranofin on the MMP values, and levels of cytochrome *c* and Bcl-2 family proteins in Hep3B cells. Cells were treated with the indicated concentrations of auranofin for 24 h. **A.** Cells were collected and incubated with 10 μM JC-1 for 20 min at 37°C in the dark. The cells were then washed with PBS, and the mean JC-1 fluorescence intensity was detected by flow cytometry. The data represent the means of two independent experiments. The cytosolic and mitochondrial (**B**), and total cellular proteins (**C**) were extracted and separated by SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis using the indicated antibodies and an ECL detection system. Equal protein loading was confirmed by analysis of actin in the protein extracts.

(Fig. 3A), suggesting that auranofin depolarized the mitochondrial membrane. A loss of MMP promotes the release of pro-apoptotic proteins, such as cytochrome *c,* into the cytosol (Scorrano and Korsmeyer 2003), so we compared the levels of cytochrome *c* in the cytosolic and mitochondrial fractions of auranofin-treated Hep3B cells. As shown in Fig. 3B, auranofin promoted a concentration-dependent increase in the release of cytochrome *c* from the mitochondria into the cytosol.

The Bcl-2 family proteins play a crucial role in mitochondria homeostasis (Richardson and Kaye 2008), so we also monitored the effect of auranofin on the levels of Bcl-2 family proteins. Auranofin treatment reduced the levels of anti-apoptotic Bcl-2 protein, but increased those of the pro-apoptotic Bax protein in Hep3B cells (Fig. 3C). Auranofin therefore could induce the mitochondrial dysfunction, followed by loss of the MMP and release of cytochrome *c* into the cytosol, resulting in the activation of the intrinsic pathway.

Auranofin modulates the expression of death receptor (DR)--related proteins and Bid in Hep3B cells

We explored a potential mechanism for auranofin-induced apoptosis in Hep3B cells by examining the effects of auranofin on the expression of DR-related proteins. As shown in Fig. 4A, auranofin treatment increased the expression of DR4 and DR5 in a concentration-dependent manner, whereas the levels of tumor necrosis factor related to the apoptosis-inducing ligand (TRAIL) were relatively unchanged by auranofin treatment. In addition, auranofin treatment decreased the level of full-length Bid protein, a BH3 interacting domain death agonist, presumably resulting from truncation by the activated caspase-8 (Billen et al. 2008; Kantari and Walczak 2011). A progressive accumulation of truncated Bid (tBid) was observed (Fig. 3C), suggesting the possible involvement of both the extrinsic and the intrinsic apoptosis pathways in auranofin-induced apoptosis in Hep3B cells.

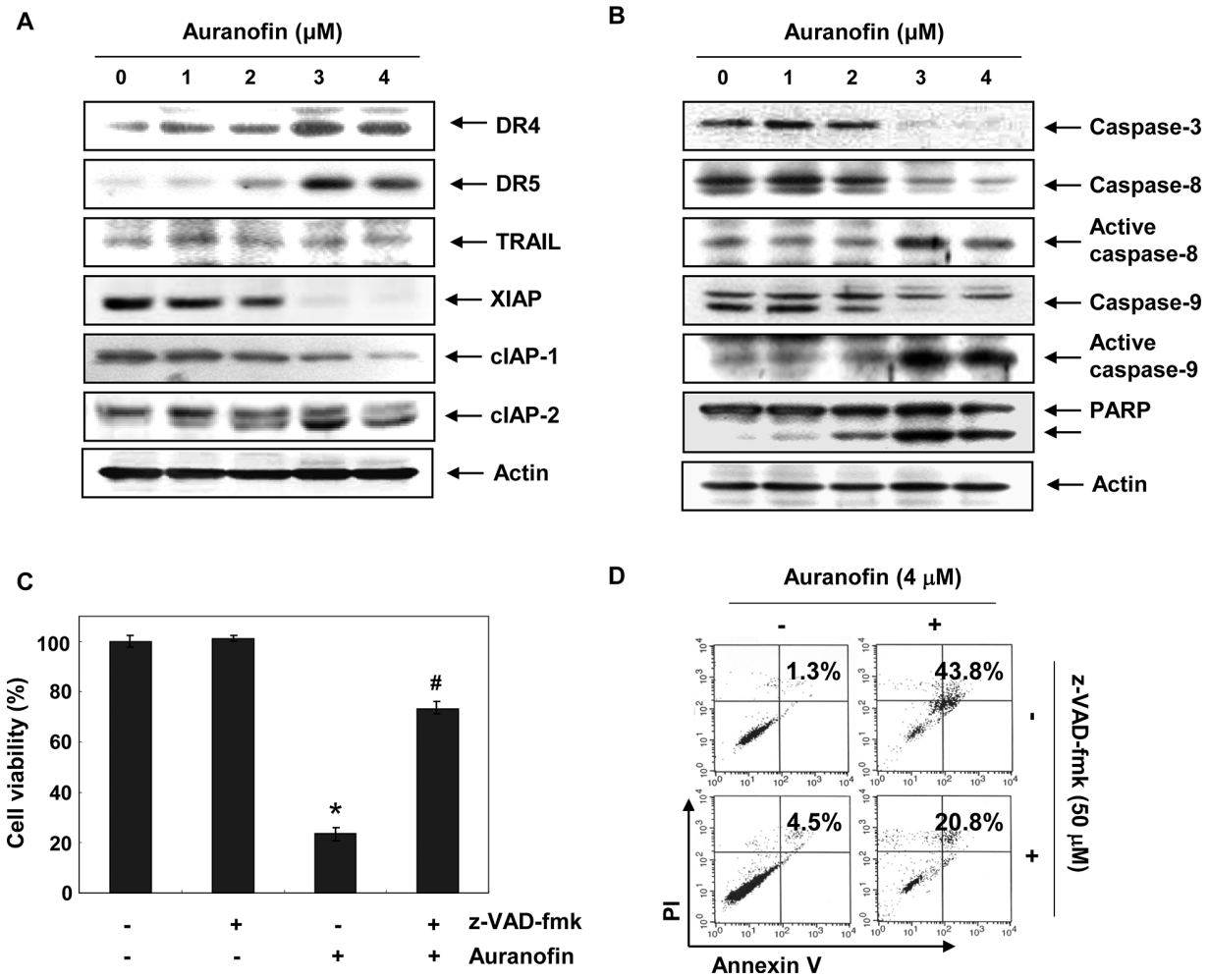

Figure 4. Effects of auranofin on the levels of DR-related and IAP family proteins (**A**), and caspases in Hep3B cells (**B**). After 24 h incubation with the indicated concentrations of auranofin, the cells were lysed, and cellular proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control. **C**. Cell viability was determined by the MTT assay. **D**. The percentage of apoptotic cells (Annexin V^+/PI^- cells) was analyzed by flow cytometry. The data are the means of the two different experiments. Each point represents the mean ± SD of three independent experiments (* $p < 0.05$ *vs.* untreated control; # $p < 0.05$ *vs.* auranofin-treated cells).

Auranofin induces caspase-dependent apoptosis in Hep3B cells

The expression levels of caspases in auranofin-treated Hep3B cells were measured in order to elucidate the role of caspases in auranofin-induced apoptosis. As shown in Fig. 4B, treatment with auranofin decreased the pro-forms of caspase-8 and -9, which are initiator caspases of the extrinsic and intrinsic apoptosis pathways, respectively. The levels of caspase-3, an effector caspase, were also decreased, but the active forms of caspase-3 were not detected. Auranofin increased the active forms of caspase-8 and -9 in a concentration-dependent manner, and increased the levels of the cleaved form of poly (ADP-ribose) polymerase (PARP), which is the cellular substrate of activated caspase-3 (Lazebnik et al. 1994; Agarwal et al. 2009). The expression levels of inhibitor of the apoptosis proteins (IAP) family proteins such as XIAP, cIAP-1 and cIAP-2 were suppressed or the proteins cleaved in auranofin-treated Hep3B cells (Fig. 4A).

The contribution of caspases to auranofin-induced apoptosis was demonstrated by pretreating Hep3B cells with the pan-caspase inhibitor, z-VAD-fmk, for 1 h, followed by incubation with auranofin for 24 h. Flow cytometry analysis and MTT assays revealed that pre-treatment with z-VAD-

fmk significantly prevented the loss of cell viability and the accumulation of annexin V positive cells in auranofin-treated Hep3B cells (Fig. 4C and D), suggesting that auranofin treatment induces caspase-dependent apoptosis in Hep3B cells.

Auranofin triggers apoptosis through ROS generation in Hep3B cells

The possible relationship between auranofin-induced apoptosis and ROS generation and TrxR inactivation was examined by measuring ROS production. The maximum generation of ROS in response to auranofin was observed at 1 h in Hep3B cells; however, this heightened ROS level was completely abrogated by prior treatment of the cells with NAC, a well-known ROS scavenger (Fig. 5A). The possibility that ROS participates in auranofin-induced apoptosis was tested by examining the effects of NAC on the changes of apoptosis-related proteins in auranofin-treated Hep3B cells. As shown in Fig. 5B, pretreatment with NAC completely abolished the auranofin-induced increases of DRs and Bax and inhibited the down-regulation of IAP family proteins and the activation of caspases in auranofin-treated Hep3B cells (Fig. 5B and C).

Inhibition of ROS generation by NAC markedly inhibited the accumulation of apoptotic cells and the formation of DNA fragments when compared to cells treated with auranofin alone (Fig. 6A and B). Pretreatment with NAC also completely blocked the auranofin-induced loss of cell viability, whereas the auranofin-induced inactivation of TrxR was dramatically restored by obstruction of ROS generation in the presence of NAC (Fig. 6C and D). Therefore, auranofin appeared to inhibit TrxR activity in a ROS-dependent fashion and the generation of ROS was necessary for the auranofin-induced apoptosis in Hep3B cells.

Discussion

Auranofin, a drug used to treat rheumatoid arthritis, is a potent inhibitor of mitochondrial TrxR, since it can block

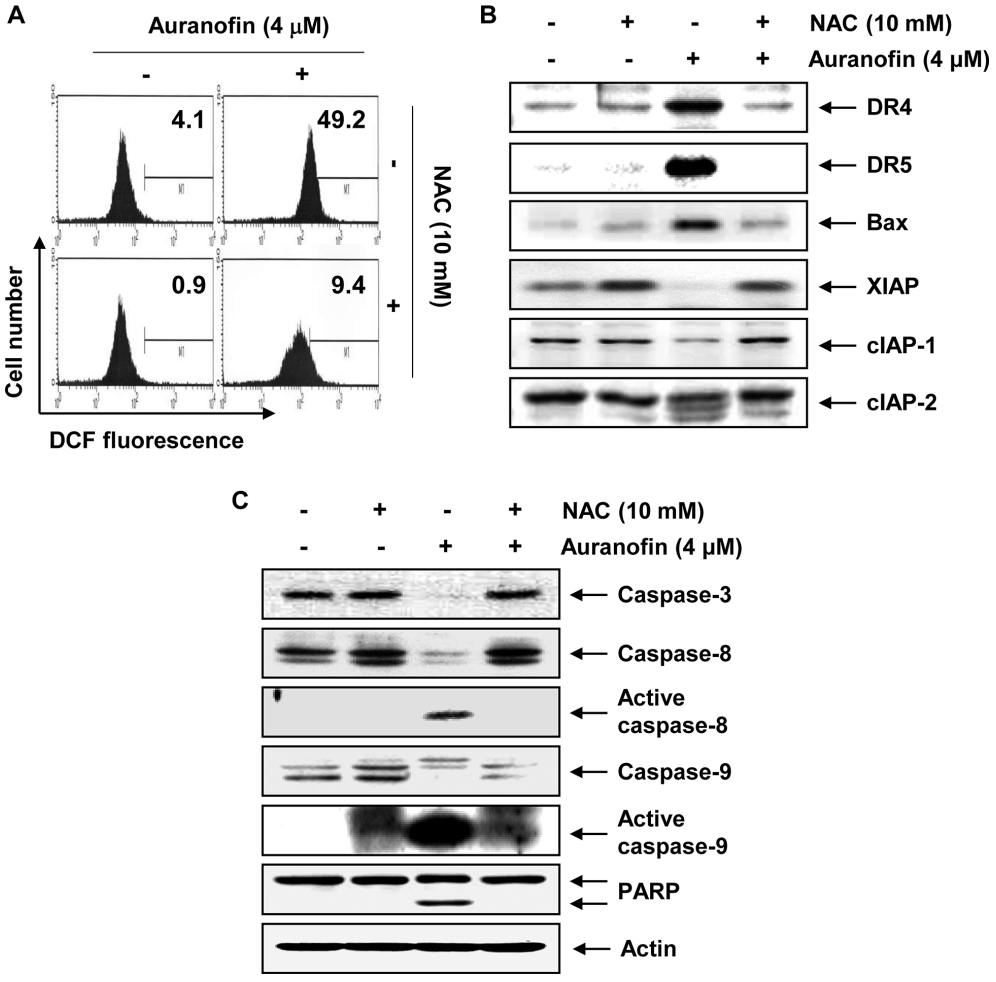

Figure 5. Effects of a ROS scavenger, NAC (N-acetyl-L-cysteine), on the modulation of apoptosis-related proteins by auranofin in Hep3B cells. **A.** Cells were either treated with 4 μg/ml auranofin for 1 h or pretreated with NAC (10 mM) for 2 h before auranofin treatment and then collected. The medium was discarded, and the cells were incubated at 37°C in the dark for 20 min with new culture medium containing 10 μM DCFH-DA. ROS generation was measured by flow cytometry. **B.** and **C.** The cells were pretreated with 10 mM NAC for 1 h before 4 μg/ml auranofin treatment. After a 24 h incubation, total proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an ECL detection system. Actin was used as an internal control.

its active site (Rigobello et al. 2002; Craig et al. 2012). Much recent evidence now indicates that auranofin also inhibits cell proliferation in various cancer cell lines by inducing apoptosis (Liu et al. 2000; Marzano et al. 2007; Gandin et al. 2010; Fiskus et al. 2014; Park et al. 2014b; Varghese and Büsselberg 2014; Zou et al. 2015). However, the relationship of the effects of auranofin on apoptosis induction and ROS generation in cancer cells and the underlying detailed mechanisms have not yet been established. The present study describes the anti-cancer effects and cellular events underlying the growth inhibitory effect of auranofin in human hepatocellular carcinoma Hep3B cells.

Apoptosis in mammalian cells is mediated through two major pathways: the extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) pathways (Hale et al. 1996; Fulda and Debatin 2004). The extrinsic apoptotic pathway is initiated by interaction between death ligands and their corresponding receptors at the plasma membrane to form the death-inducing signaling complex (DISC) with its adaptor molecule, Fas associated death domain (FADD) (Debatin and Krammer 2004; Gloire et al. 2008). DISC activates caspase-8, an apoptosis initiator of the extrinsic pathway, which in turn activates the downstream executioner caspases, such as capase-3 and -7, culminating in extrinsic apoptosis. By contrast, the intrinsic pathway begins with the disruption of the MMP and the release of apoptogenic proteins, such as cytochrome *c*, from mitochondria into the cytosol. In the cytosol, cytochrome *c* can activate caspase-9, an apoptosis initiator of the intrinsic pathway, which in turn activates the effector caspases (Hengartner 2000; Fulda and Debatin 2004). Following the activation of effector caspases, several substrate proteins, including PARP, are cleaved, eventually triggering apoptosis (Lazebnik et al. 1994; Agarwal et al. 2009). Therefore, the activation of caspase-8 and -9 plays a key role in the initiation of extrinsic and intrinsic pathways, respectively.

The present study confirmed an apparent activation of caspase-8 in auranofin-treated Hep3B cells, which was associated with the induction of DRs (Fig. 4A and B). In addition, auranofin concentration-dependently activated caspase-9 and -3, concomitant with the degradation of PARP. These observations suggest that auranofin induces apoptosis in Hep3B cells through the activation of both the intrinsic and the extrinsic pathways. Auranofin-induced Hep3B cell apoptosis was also accompanied by decreased levels of IAP family proteins, which bind with caspases and then suppress their activation (Roy et al. 1997; Deveraux et al. 1998). The auranofin-induced growth reduction and apoptosis was significantly attenuated in the presence of a pan-caspase inhibitor (Fig. 4C and D), indicating that activation of caspases was a critical step in auranofin-induced apoptosis in Hep3B cells.

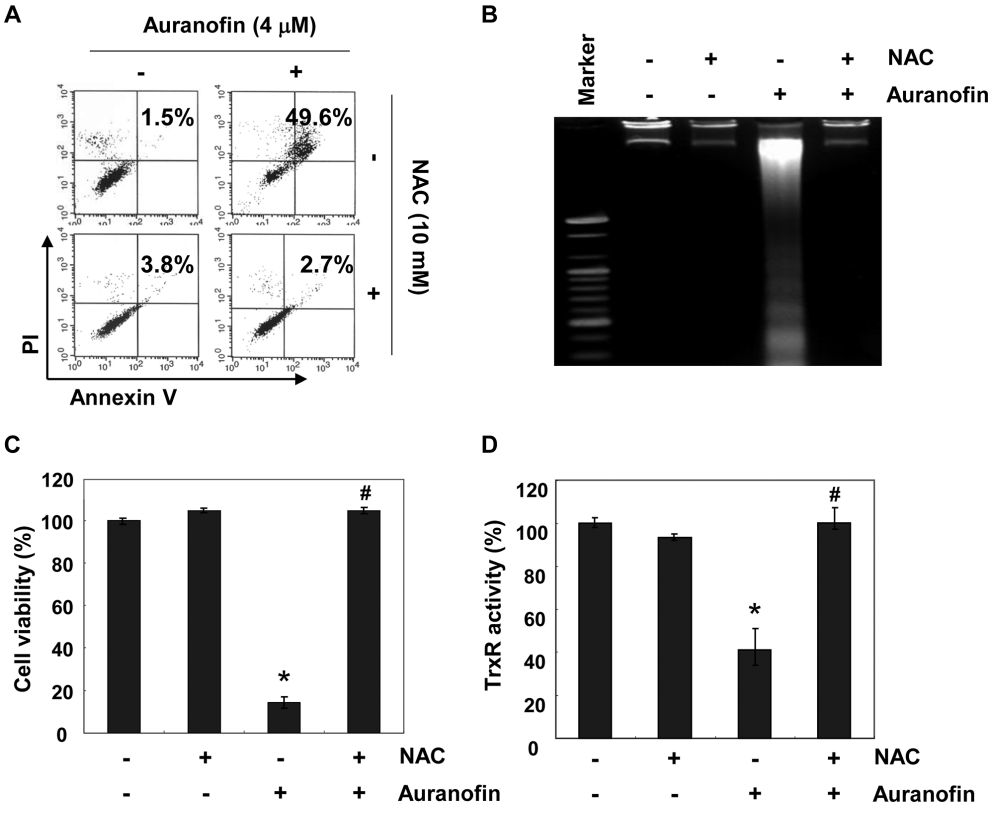

Figure 6. Induction of ROS-dependent apoptosis by auranofin in Hep3B cells. Cells were pretreated with 10 mM NAC for 1 h before 4 μg/ml auranofin treatment for 24 h. **A.** The percentage of apoptotic cells (Annexin V^+/PI^- cells) was analyzed by flow cytometry. The data are the means of the two different experiments. **B.** DNA fragmentation was analyzed by extracting genomic DNA, electrophoresis in a 1.5% agarose gel, and then visualizing by EtBr staining. **C.** Cell viability was determined by the MTT assay. **D**. The TrxR activities were measured using a TrxR colorimetric assay. Each point represents the mean ± SD of three independent experiments (* $p < 0.05$ *vs.* untreated control; # $p < 0.05$ vs. auranofin-treated cells).

The caspase-cascade signaling system is also regulated by several different molecules, including members of the Bcl-2 family, which are involved in control of the process of apoptosis by interactions between pro-apoptotic and anti-apoptotic members (Stennicke and Salvesen 1999; Lavrik et al. 2005). These act on the mitochondrion to prevent or to facilitate the disruption of MMP and release of apoptogenic factors (Scorrano and Korsmeyer 2003; Breckenridge and Xue 2004). In addition, the caspase-8-mediated cleavage of the pro-apoptotic protein Bid generates tBid, a truncated form of Bid, which translocates to the mitochondria to promote the cytosolic release of cytochrome *c* to initiate the intrinsic pathway (Billen et al. 2008; Kantari and Walczak 2011). Auranofin-induced apoptosis in Hep3B cells was accompanied by the down-regulation of Bcl-2, up-regulation of Bax, and the loss of MMP when compared to untreated cells (Fig. 3). Auranofin treatment also increased the expression of tBid and induced the release of cytochrome *c* into the cytosol in Hep3B cells (Fig. 3). Therefore, auranofin most likely caused Bid cleavage to yield truncated Bid by activating caspase-8, which in turn would activate the mitochondrial pathway of apoptosis. Caspase-8 would therefore appear to act as an upstream executor of the mitochondrial apoptotic pathway in auranofin-treated Hep3B cells.

ROS play an important role as secondary messengers in the regulation of cellular functions; however, excessive production of ROS can cause the loss of MMP and induce apoptosis by releasing cytochrome *c* from the mitochondria to the cytosol (Matés and Sánchez-Jiménez 2000; Wu 2006). Therefore, the generation of ROS may contribute to mitochondrial damage and lead to cell death by acting as apoptotic signaling molecules (Pelicano et al. 2004; Ryter et al. 2007). ROS-mediated mitochondrial dysfunction has been demonstrated as an critical mediator of auranofin-induced apoptosis in certain cancer cell lines (Liu et al. 2000; Rigobello et al. 2002; Marzano et al. 2007; Pessetto et al. 2013; Fiskus et al. 2014; Zou et al. 2015), but the roles of ROS in modulating TrxR activity in auranofin-induced cancer cell apoptosis have not been established. Auranofin influenced the level of ROS in Hep3B cells stained with DCF-DA, with auranofin-induced ROS generation evident as early as 1 h after treatment (Fig. 5A). However, blocking the generation of ROS with the antioxidant NAC decreased the intracellular ROS levels (Fig. 5A) and conferred protection against the auranofin-induced increase in DRs and Bax (Fig. 5B). In addition, NAC pretreatment attenuated the activation of caspases and degradation of PARP (Fig. 5C). The blocking of ROS generation markedly inhibited auranofin-induced apoptosis and growth inhibition (Fig. 6A–C), and was associated with the restoration of auranofin-inactivated Trx activity in Hep3B cells (Fig. 6D).

In conclusion, auranofin, an inhibitor of TrxR, induced the extrinsic and intrinsic apoptosis pathways in hepatocellular carcinoma Hep3B cells by targeting TrxR. Further studies are warranted to elucidate the detailed mechanism underlying the ROS-mediated inactivation of Trx activity. Nevertheless, the data presented here support a requirement for ROS generation in the auranofin-induced inactivation of Trx activity, as well as in the anti-cancer effects observed in Hep3B cells. Taken together, our results indicate that auranofin may be an important therapeutic agent for cancer treatment.

Conflict of interest. The authors have no conflict of interest to declare.

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