The role of NF-κB in PARP-inhibitor-mediated sensitization and detoxification of arsenic trioxide in hepatocellular carcinoma cells

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(Received December 21, 2014; Accepted March 5, 2015)

ABSTRACT — The therapeutic efficacy of arsenic trioxide (ATO) for treatments of solid tumors is restricted by its drug resistance and chemotoxicity. In this study, we investigated ATO sensitization and detoxification effect of the Poly (ADP ribose) polymerase-1 (PARP-1) inhibitor 4-Amino-1,8-naphthalimide (4AN) in the hepatocellular carcinoma cell line HepG2. We firstly reported that ATO treatment induced the activation of Nuclear factor of κB (NF-κB) and its downstream anti-apoptosis and pro-inflammatory effectors in a PARP-1-dependent manner and thus conferred HepG2 cells with ATO resistance and toxicity. 4AN significantly suppressed the ATO-induced NF-κB activation, which promotes the apoptotic response and alleviates the inflammatory reaction induced by ATO, resulting in sensitization and detoxification against ATO. We also demonstrated that the ATO-induced activation of PARP-1 and NF-κB was closely associated with the oxidative DNA damage mediated by the generated reactive oxygen species (ROS). Furthermore, the attenuation of ATO-induced ROS and the resulting oxidative DNA damage by N-acetyl-L-cysteine (NAC), a potent antioxidant, significantly reduced the activation of PARP-1 and NF-κB in ATO-treated cells. Our study provides novel insights into the mechanism of the PARP-1-mediated NF-κB signaling pathway in ATO resistance and toxicity in anticancer treatments. This study also highlights the application potential of PARP-1 inhibitors in ATO-based anti-cancer treatments and in prevention of NF-κB-mediated therapeutic resistance and toxicity.

Key words: Arsenic trioxide, Poly (ADP ribose) polymerase-1, Nuclear factor of κB, Reactive oxygen species, Oxidative DNA damage

INTRODUCTION

Arsenic trioxide (ATO), a traditional Chinese medicine, is an effective drug for treatments of hematological malignancies, particularly acute promyelocytic leukemia (APL), with a complete remission rate of up to 90% (Liu et al., 2012; Wang and Chen, 2008). Numerous studies demonstrated that ATO can also inhibit the growth of various solid tumor cell lines, including hepatocellular carcinoma, ovarian cancer, gastric cancer, and esophageal cancer (Xia et al., 2012; Liu et al., 2011). Among multiple molecular bases proposed for the anti-tumor effect of ATO, ROS-mediated DNA damage and apoptosis induction are considered the central mechanisms (Liu et al., 2011, 2012). However, in most phase II clinical trials, the curative benefit of ATO against solid tumors is much less effective than that against APL because its dosage required to exert anticancer effect is higher than those required in hematologic malignancies (Lin et al., 2007; Liu et al., 2013). High doses of ATO are prone to cause severe toxic effects, including hepatotoxicity, cardiotoxicity, nephrotoxicity, and even sudden death (Hao et al., 2013; Vineetha et al., 2014). Therefore, dose-dependent toxicity and drug resistance considerably restrict ATO application for treatment of solid tumors. Novel strategies aimed at reducing ATO-induced drug resistance and toxicity without affecting its anti-tumor effects are of urgent significance for development of ATO-based anticancer therapies.

Poly (ADP ribose) polymerase-1 (PARP-1), a well-known DNA damage sensor protein, is involved in the recruitment and assembly of DNA repair proteins in...
response to DNA damage (Javle and Curtin, 2011). It is generally considered that the activation of PARP-1 directly enhances DNA repair in cancer cells and thus causes tumor resistance to chemo- and radiotherapeutics. In this context, PARP-1 inhibitors have been developed and widely used to potentiate the anti-tumor efficacy of a number of chemotherapeutic agents, including irradiation, temozolomide, irinotecan, and topotecan (Mégnin-Chanet et al., 2010). More importantly, PARP-1 inhibitors can also attenuate toxicities caused by chemotherapeutic agents, such as cisplatin nephrotoxicity and doxorubicin cardiomyotoxicity (Mukhopadhyay et al., 2009). Among these factors, NF-κB is a stress-inducible transcription factor that is inactive in the cytoplasm. In response to stimuli, such as DNA damage and/or immune factors, NF-κB translocates into the nucleus and transactivates its downstream genes, including anti-apoptotic genes, such as Bcl-2, Bcl-XL, and XIAP, and pro-inflammatory genes, such as iNOS and ICAM-1 (Jing and Lee, 2014). NF-κB is constitutively active in hematologic malignancies and various solid tumors, such as hepatocellular carcinoma (Prasad et al., 2010). This is proposed to be associated with poor prognosis, high malignancy, and therapeutic resistance to cancer treatments in clinics (Prasad et al., 2010). Moreover, many anticancer agents trigger NF-κB in tumor cells, which has been demonstrated in most processes, to make cells escape from apoptosis, thereby conferring chemo- and radioresistance to cancer cells (Erlstad and Cusack, 2013; Bailey et al., 2014; Ma et al., 2011). Numerous studies also documented the relationship between chemotherapeutic toxicity and acute inflammatory responses (Leung et al., 2014). Given that acute inflammatory responses are mainly mediated by NF-κB (Jing and Lee, 2014), it is conceivable that NF-κB may play a major role in the occurrence and progression of chemotherapeutic toxicity. This notion has been substantiated by the protective effects of anti-inflammatory drugs targeting the NF-κB pathway, such as aspirin and chrysin, against chemotoxicity, including DOX-induced cardiotoxicity (Mantawy et al., 2014).

In 2000, the interaction of PARP-1 with NF-κB was first reported when a binding site of PARP-1 was identified in NF-κB (Pleschke et al., 2000). Later, PARP-1 was found to function as a novel co-activator of NF-κB in inflammatory disorders, including colitis and drug-induced toxicity (e.g., kidney and liver injury) (Castrì et al., 2014). Inhibition of PARP-1 with the use of inhibitors showed a positive therapeutic outlook in amelioration of such disorders via suppression of NF-κB-mediated inflammatory response (Kapoor et al., 2014; Wang et al., 2013b). In the past decade, due to the emphasis on anti-apoptosis signaling pathways mediated by NF-κB, numerous studies reported that PARP-1 inhibitors exhibit their sensitization effect on anticancer treatments, such as irradiation and adriamycin, by inhibiting NF-κB (Anto et al., 2003; Hunter et al., 2012; Jing and Lee, 2014). These findings suggested that the NF-κB pathway is actively involved in the chemosensitization and chemodetoxification effects of PARP-1 inhibitors.

ATO can activate NF-κB in many cell models, such as mouse epidermal Cl41 cells and human leukemia Jurkat cells (Ouyang et al., 2006; Wang et al., 2013c). Some recent studies also revealed that arsenic compounds can disturb PARP-1 expression in the human keratinocyte cell line HaCat (Qi et al., 2012; Wang et al., 2013c; Ding et al., 2009). Despite numerous studies have been performed, the interaction of PARP-1 and NF-κB in response to ATO remains unknown. Furthermore, whether the inhibition of PARP-1 can improve the anticancer efficacy of ATO via alterations in the NF-κB pathway needs to be explored. Therefore, in this study, we used a hepatocellular carcinoma cell line, HepG2, to investigate ATO sensitization and detoxification effect of PARP-1 inhibitor, with a special emphasis on its interference with NF-κB signal pathway. 4-Amino-1,8-naphthalimide (4AN), a traditional PARP-1 inhibitor, was utilized to suppress PARP-1 activity. Given that PARP-1 is an important co-activator of NF-κB and PARP-1 is a key protein in responses to oxidative DNA damage triggered by ATO, we hypothesized that ATO-induced ROS may lead to oxidative DNA damage and the resulting PARP-1 activation. The activated PARP-1 further triggers NF-κB and transactivates its downstream anti-apoptotic and pro-inflammatory effectors, leading to drug resistance and toxicity in ATO treatment. Accordingly, the inhibition of PARP-1 with 4AN may block the activation of NF-κB and its downstream effectors; this blockage enhances the apoptotic response and reduces the inflammatory reaction induced by ATO, resulting in sensitization and detoxification against ATO.
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To test these hypotheses, we designed and conducted a series of experiments in this study.

MATERIALS AND METHODS

Cell culture and reagents

The human liver hepatocellular carcinoma cells (HepG2) were purchased from the Type Culture Collection of the Chinese Academy of Science, Wuhan, China. The cells were routinely cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 unit/mL) and streptomycin (100 μg/mL). ATO was purchased from YiDa Pharmaceutical Co. Ltd., Harbin Medical University, Heilongjiang, China. PARP-1 inhibitor 4AN was from Alexis, Lausen, Switzerland. NF-κB activator lipopolysaccharide (LPS), NF-κB inhibitor pyrrolidine dithiolar-bamate (PDTC) and anti-oxidant N-acetyl-L-cysteine (NAC) were all purchased from Sigma, Louis, MO, USA. 4AN and PDTC were dissolved in dimethylsulfoxide (DMSO). Therefore, cells treated with DMSO were served as control in some indicated experiments in our study.

Cell viability measured by MTT assay

The cell viability was determined by 3-(4,5-dimethyl thiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as previously described with some modifications (Luo et al., 2012). Briefly, HepG2 cells were seeded in 96-well plates at a density of 2 × 10^4 cells (200 μL) per well. After overnight incubation, cells were treated with ATO (range from 0-200 μM), ATO combined with LPS (10 μg/mL), ATO combined with PDTC (10 μM), ATO with 4AN (10 μM), and ATO with 4AN (10 μM) and LPS (10 μg/mL). Subsequently, 100 μL of 0.5 mg/mL MTT solution (Amresco, Solon, OH, USA) was added into each well, and cells were incubated at 37°C for additional 4 hr. Next, 100 μL DMSO was used to dissolve formazan crystals. Absorbance at 570 nm was measured by a Bio-Rad micro-plate reader (Bio-Rad, Hercules, CA, USA). The blank wells were set as the zero point, and the cells with DMSO were used as control. Cell viability was calculated using the formula: Cell viability (%) = (A570 of treatment group-A570 of blank group) / A570 of control group-A570 of blank group) × 100, while 50% inhibitory concentration (IC_{50}) was obtained by probit analysis.

Detection of apoptosis by Annexin V-FITC/ PI staining

The level of apoptosis was examined using AnnexinV-fluorescein isothiocyanate (FITC) and propidium iodide (PI) dual staining. Briefly, cells were cultured in 6-well plates at a density of 1 × 10^4 cells per well and treated with 10 μM ATO alone, 10 μM ATO with 10 μg/mL LPS, 10 μM ATO with 10 μM PDTC, 10 μM ATO with 10 μM 4AN, and 10 μM ATO with 10 μM 4AN and 10 μg/mL LPS for 24 hr. Cells treated with DMSO were used as controls. After treatment, the cells were collected by trypsinization and centrifugation at 110 × g for 5 min at 4°C, and then resuspended in binding buffer containing 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of propidium iodide (PI). After incubation at 37°C in the dark for 30 min, cells were subjected to flow cytometry (Beckman Coulter, Indianapolis, IN, USA). 20,000 cells from each sample were analyzed by the software of Windows Multiple Document Interface for Flow Cytometry, version 2.8 (The Scripps Research Institute, San Diego, CA, USA).

Western blotting

In brief, cells were treated with indicated dosages of ATO alone (10 μM), ATO along with 4AN (10 μM) or ATO in combination with NAC (10 mM) for 12 or 24 hr. The cells were harvested, homogenized and lysed. Then the total protein, nuclear protein and cytoplasm protein were extracted using a commercial kit (KeyGEN, Nanjing, China) respectively. Proteins in the same amount (60 μg) were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking of non-specific epitopes for 2 hr at room temperature with 5% nonfat dry milk, the PVDF membranes were incubated with a primary antibody specific against PARP-1 (1:500) (ABclonal, Cambridge, MA, USA), NF-κB (p65) (1:500) (ABclonal Biotech Co., Ltd), Bax (1:500) (Boster Bio, Wuhan, China), Bcl-2 (1:500) (Bax (1:500) (Boster Bio), iNOS (1:1000) (Boster Bio), as well as Lamin B (1:1000) and β-actin (1:1000) (ZSGB Bio, Beijing, China) overnight at 4°C. After that, the PVDF membranes were incubated with a horseradish peroxidase conjugated secondary antibody (1:5000) (Millipore) at room temperature for 1 hr. Subsequently, the PVDF membranes were incubated with an enhanced chemiluminescent substrate (Millipore) and the chemiluminescent signals were visualized and imaged with a Molecular Imager Gel Doc XR System (Millipore). The intensities of chemiluminescence were quantified by the Quantity One software (Bio-Rad). The values of nucleoprotein (nuclear p65 and PARP-1) were normalized for the Lamin B expression level, whereas the values of total protein (iNOS, Bcl-2, Bax) and cyto-
plasma protein (cytoplasm p65) were normalized for the β-actin expression level.

**Measurement of NF-κB nuclear translocation by immunofluorescence**

NF-κB activation, showed as p65 nuclear translocation, was detected by an immunofluorescence-labeled assay using a Cellular NF-κB Translocation Kit (Beyotime Biotech, Shanghai, China). In brief, cells in log phase were plated at a density of 5 × 10⁶ cells/well with sterilized 24 mm × 24 mm slides at the bottom of each well. After overnight culture for adhesion, the cells were treated by 10 μM ATO, with or without 10 μM, for 24 hr. Cells treated with DMSO were employed as controls. Then cells were washed twice and fixed for 15 min at room temperature, and subsequently incubated with a blocking buffer for 1 hr to suppress non-specific binding. After that, cells were incubated with a primary antibody specific against NF-κB (p65) for 1 hr, followed by incubation with a Cy3-conjugated secondary antibody for another 1 hr. Then cells were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min, then visualized and imaged under a fluorescent microscope at 400 × magnification (Eclipse TiU, Nikon Corp., Tokyo, Japan). Cell nuclear and p65 protein fluoresced as blue and red, respectively. To create a two-color image, the red and blue images were merged, and the purple fluorescence was produced in areas of co-localization, which indicated the location of p65 protein in nuclear. The density of cell fluorescence was evaluated using the Image J 1.48 software (National Institutes of Health, Bethesda, MD, USA). The parameter value of average optical density (AOD) was utilized for assessment of the level of intracellular ROS and was calculated using the following formula: AOD = Sum of integrated optical density (IOD)/Sum area.

**Assessment of intracellular ROS level**

The production of intracellular ROS was detected by using an Oxidation-Sensitive Fluorescent Probe (DCFH-DA) Kit (Keygen Biotech, Nanjing, China). Briefly, 1 × 10⁶ cells were treated with DCFH-DA with a final concentration at 10 μM at 37°C in the dark for 1 hr. Then cells were washed with PBS and visualized under a fluorescence microscope (DMLB2, Leica, Wetzlar, Germany). The density of cell fluorescence was evaluated using the Image-Pro® plus 6.0 software (Media Cybemetics, Rockville, MD, USA). The parameter value of average optical density (AOD) was utilized for assessment of the level of intracellular ROS and was calculated using the following formula: AOD = Sum of integrated optical density (IOD)/Sum area.

**Detection of oxidative DNA damage using an ELISA assay**

Oxidized guanines including 8-hydroxyguanosine (8-OHG) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), is the most common DNA oxidant damage and thus are widely used as the biomarker of DNA oxidative damage. Here, we detected the cellular oxidized guanine by using an ELISA assay. Briefly, 1 × 10⁶ cells were treated with ATO alone (10 μM), ATO (10 μM) combined with 4AN (10 μM) or ATO (10 μM) and NAC (10 mM) for 24 hr. Untreated cells were utilized as controls. Then cells were harvested for extracting DNA using a commercially extraction kit (Aid, Beijing, China), according to the manufacturer’s protocol. The purified DNA (50 μL) was directly used to detect oxidized guanine including 8-OHG and 8-OHdG by using a DNA Oxidative damage ELISA kit (Cayman, Rockford, IL, USA), following the manufacturer’s instructions. Absorbance at 405 nm was measured by a Bio-Rad micro-plate reader (Bio-Rad). The concentration of oxidized guanine was calculated from standard curves obtained from freshly prepared standard solutions, and calculated using a computer spreadsheet provided by the manufacture (https://www.caymanchem.com/msdss/589320m.pdf).

**Statistical methods**

All experiments were conducted independently at least three times. Data were expressed as mean ± standard deviation (S.D.). Statistical analysis was performed by SPSS 13.0 software. One-way analysis of variance (ANOVA) was used to evaluate significant difference among multiple experimental groups, and the least-significant difference (LSD)-t test and the Dunnett-t test were combined for significant difference detection. Statistical comparisons of the 50% inhibitory concentration (IC₅₀) among the different treating groups were analyzed by Student’s t-test. P < 0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**PARP-1 inhibitor 4AN sensitized ATO in HepG2 cells, and this sensitization effect was associated with NF-κB suppression**

The viability of HepG2 cells under ATO treatment was detected using MTT assay. First, we examined the func-
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NF-κB-dependent ATO sensitization by 4AN was achieved by enhanced apoptosis induction

ATO can inhibit the growth of various cancer cells by inducing apoptosis (Liu et al., 2011, 2012). By functioning as an anti-apoptosis factor, the aberrant activation of NF-κB contributes to the initiation and progression of chemoresistance. Accordingly, loss or inhibition of NF-κB leads to chemosensitization in a variety of tumors (Erstad and Cusack, 2013; Bailey et al., 2014; Ma et al., 2011). In this regard, NF-κB-dependent ATO sensitization by the PARP-1 inhibitor 4AN may result from the elevated apoptotic cell death. To test this possibility, we detected the level of apoptosis by using Annexin V-FITC/PI assay.

In this study, 10 μM ATO was selected to induce apoptosis on the basis of the following: 1) this dosage has been used in numerous studies to determine the mechanism of the anticancer activity of ATO (Walker et al., 2010; Wang et al., 2012a); and 2) 10 μM ATO is used in clinics for cancer chemotherapy because pharmacological studies demonstrated that it exhibits effective anti-tumor efficacy in many cancer cells, such as HepG2 cells, breast cancer cells, and lung carcinoma cells (Sun et al., 2011; Walker et al., 2010; Wang et al., 2012a). Our results also showed that 10 μM ATO exerted anti-cancer effect on HepG2 cells (Fig. 1). Therefore, 10 μM ATO could be suitable in determining the mechanism by which 4AN could sensitize ATO in HepG2 cells.

The results of Annexin V-FITC/PI assay indicated that the NF-κB activator LPS decreased the percentage of apoptotic cells in ATO-treated HepG2 cells (17.43% ± 3.15% in the ATO group versus 30.16% ± 5.08% in the ATO+LPS group) (P = 0.035). Conversely, the NF-κB inhibitor PDTC remarkably promoted the apoptotic response (17.43% ± 3.15% in the ATO group versus 30.16% ± 5.08% in the ATO+PDTC group) (P < 0.001). These findings suggested that NF-κB activation could result in ATO resistance in HepG2 cells via inhibition of apoptosis, which were consistent with previous reports (Erstad and Cusack, 2013; Bailey et al., 2014; Ma et al., 2011). More importantly, HepG2 cells co-treat-
ed with ATO+4AN presented a significantly higher percentage of apoptotic cells than those treated with ATO alone (17.43% ± 3.15% in the ATO group versus 28.33% ± 4.57% in the ATO+4AN group) \((P < 0.001)\) (Fig. 2). Hence, 4AN-mediated ATO sensitization could mainly result from the enhanced cellular apoptosis. However, apoptosis in HepG2 cells treated with ATO+4AN was suppressed by LPS (28.33% ± 4.57% in the ATO+4AN group versus 19.34% ± 2.86% in the ATO+4AN+LPS group) \((P = 0.048)\) (Fig. 2), further indicating that 4AN may promote ATO-induced apoptosis by inhibiting NF-κB.

**4AN inhibited ATO-induced activation of NF-κB and its downstream effectors involved in anti-apoptosis and pro-inflammation in HepG2 cells**

NF-κB is a complex consisting of homo- and heterodimers of the Rel family members, namely, RelA...
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Fig. 2. NF-κB-mediated ATO sensitization by 4AN is associated with enhanced cellular apoptosis. The induction of apoptosis was evaluated through Annexin V-FITC/PI assay. Cells were subjected to the following treatments for 24 hr: 10 μM ATO alone, 10 μM ATO with 10 μg/mL LPS, 10 μM ATO with 10 μM PDTC, 10 μM ATO with 10 μM 4AN, and 10 μM ATO with 4AN and LPS. The cells treated with DMSO were used as controls. After treatment, the cells were harvested and assessed for apoptosis as described in MATERIALS AND METHODS. Panel (a) illustrates the DMSO control. Panel (b) illustrates the cells treated by ATO alone. Panel (c) illustrates the cell treated by ATO+LPS. Panel (d) illustrates the cells treated by ATO+PDTC. Panel (e) illustrates the cells treated by ATO+4AN. Panel (f) illustrates the cells treated by ATO+4AN+LPS. Panel (g) illustrates the percentage of apoptotic cells in different experimental groups. Data were obtained from three independent experiments and illustrated as means ± standard deviation. “*” indicates significant differences compared with cells treated by ATO alone and those treated by ATO+LPS, ATO+PDTC, ATO+4AN, or ATO+4AN+LPS. “#” denotes a significant difference compared with cells treated by ATO+4AN and those treated by ATO+4AN+LPS.

(p65), Rel B, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100); among which, RelA (p65) is responsible for the majority of the biological functions of NF-κB. As a stress-inducible transcription factor, p65 is inactive in the cytoplasm through interaction with its natural inhibitor, the κB (IκB) proteins (Israel, 2010). Various stimuli, such as DNA damage and immune factors, can induce the activation of p65 (Napetschnig and Wu, 2013). The signaling cascades converged at the inhibitor kappa-B kinase (IKK) complex can induce the phosphorylation and degradation of IκB, thereby promoting p65 nuclear translocation; this translocation is a well-accepted indicator of NF-κB acti-
To further investigate the interaction of PARP-1 and p65 nuclear translocation after ATO treatment, we determined their time-dependent alterations by using western blot. The protein expression levels of PARP-1, nuclear p65, and cytoplasmic p65 induced by 10 μM ATO was monitored up to 24 hr. In response to ATO, p65 expression time-dependently increased in the nucleus but decreased in the cytoplasm, indicating that p65 translocated from the cytoplasm into the nucleus after the treatment (Figs. 3a and 3b). Concomitant with increased p65 nuclear translocation, the expression level of PARP-1 also elevated with prolonged ATO treatment time (Figs. 3a and 3b). This finding demonstrated that PARP-1 activation may be involved in ATO-induced p65 nuclear translocation.

Based on these observations, we further investigated whether PARP-1 inhibition by 4AN affects p65 nuclear translocation after ATO treatment through western blot assay. Treatment with 10 μM ATO for 24 hr was used to efficiently activate PARP-1 and P65 nuclear translocation. As shown in Figs. 4a and 4b, the inhibition of PARP-1 by 4AN remarkably suppressed p65 nuclear translocation induced by ATO. The levels of nuclear p65 (86.73% ± 8.10% in the ATO group versus 54.10% ± 7.95% in the ATO+4AN group) (P = 0.003) decreased in cells treated by ATO and 4AN, whereas cytoplasmic p65 (35.30% ± 4.94% in the ATO group versus 85.12% ± 4.31% in the ATO+4AN group) (P < 0.001) increased compared with those with ATO treatment alone.

These findings were supported through immunofluorescence assay. As shown in Figs. 4c and 4d, the average optical density (AOD) of purple fluorescence in the cells treated with ATO was significantly higher than that in the DMSO control group (0.092% ± 0.018% in the ATO group versus 0.028% ± 0.005% in the DMSO control group) (P = 0.001). As the purple fluorescence is an indicator of the location of the p65 protein into the nuclei, this finding suggested that p65 nuclear translocation was enhanced by ATO in HepG2 cells. Our data were consistent with those of previous studies (Ouyang et al., 2006; Wang et al., 2013c), but some reports showed that ATO can inhibit NF-κB in several cell models (Zhang et al., 2014). This discrepancy may be attributed to the different cell lines and model systems used. In the presence of the PARP-1 inhibitor 4AN, the AOD of purple fluorescence decreased (0.092% ± 0.018% in the ATO group versus 0.064% ± 0.012% in the ATO+4AN group) (P = 0.038), suggesting that PARP-1 inhibition could suppress the activation of NF-κB induced by ATO (Figs. 3 and 4).

Taken together, our data suggested that PARP-1 activation was required for p65 nuclear translocation in response to ATO, whereas PARP-1 inhibition by 4AN could, at least partially, block ATO-induced p65 nuclear translocation. However, it was reported that arsen-
ite inhibits PARP-1 in several oncogenicity studies (Qin et al., 2012; Wang et al., 2013a; Ding et al., 2009). This may be explained as follow: in studies reporting that arsenite inhibits PARP-1 (Qin et al., 2012; Wang et al., 2013a; Ding et al., 2009), the researchers investigated the chronic toxicity and carcinogenicity of sodium arsenite; this compound is a form of trivalent arsenic with a molecular formula of NaAsO2 and is an environmental pollutant associated with a wide range of human disorders. To explore the chronic toxicity of sodium arsenite, these studies conducted long-term (3-90 d) and low-dose (0.1-2 μm) sodium arsenite treatments. PARP-1 suppression in response to low doses of sodium arsenite may be the long-term tolerance results. However, in the present study, we explored the role of PARP-1 in the anticancer activity of ATO, which is another form of trivalent arsenic (molecular formula = As2O3). For this purpose, treatments with a relatively high dose of ATO (10 μm) within a short period (12-24 hr) were carried out based on clinical applications and previous reports (Sun et al., 2011; Walker et al., 2011).
Given that PARP-1 activation is a protective mechanism of cells in response to acute DNA lesions, and that ROS-mediated DNA damage induction is an essential mechanism underlying the anti-cancer activity of ATO (Liu et al., 2011, 2012), it is conceivable that the observed PARP-1 activation could be the cellular protective mechanism in response to ATO-induced DNA damage.

4AN inhibited the activation of NF-κB downstream effectors involved in anti-apoptosis and pro-inflammation, thus conferring ATO sensitization and detoxification in HepG2 cells

NF-κB activation inhibits cellular apoptosis and promotes inflammation by inducing its downstream genes responsible for anti-apoptotic cell death and pro-inflammation, such as Bcl-2 and iNOS, respectively (Jahani-Asl and Bonni, 2013; Bai and Wang, 2014). The inhibitory effects of the PARP-1 inhibitor 4AN on NF-κB could result in alterations in NF-κB-regulated genes involved in anti-apoptosis and pro-inflammation; these alterations could promote apoptotic responses and alleviate inflammatory reaction triggered by ATO, ultimately leading to ATO sensitization and detoxification. To test this hypothesis, we used western blot analysis to detect the protein expression levels of Bcl-2 and iNOS, which are two well-known NF-κB-regulated effectors that mediate anti-apoptotic and pro-inflammatory responses, respectively. Treatment with 10 μM ATO for 24 hr was used to trigger the activation of Bcl-2 and iNOS. As expected, the expression levels of Bcl-2 and iNOS increased with elevated PARP-1 expression and p65 nuclear translocation after exposure to ATO (Figs. 4 and 5). The ATO-activated downstream effectors of NF-κB, Bcl-2, and iNOS confirmed that ATO could induce the activation of NF-κB in HepG2 cells. This finding further suggested that ATO could activate NF-κB downstream apoptotic and pro-inflammatory effectors to confer ATO resistance and toxicity upon HepG2 cells. However, the PARP-1 inhibitor 4AN significantly reduced the expression levels of Bcl-2 (71.80% ± 8.45% in the ATO group versus 42.73% ± 6.86% in the ATO+4AN group) (P = 0.001) and iNOS (46.87% ± 6.65% in the ATO group versus 14.63% ± 3.96% in the ATO+4AN group) (P < 0.001) after treatment with ATO (Figs. 5a and 5b). These findings indicated that 4AN inhibited the ATO-induced activation of NF-κB downstream effectors, which may lead to ATO sensitization and detoxification. The inhibitory effects of 4AN on NF-κB downstream proteins involved in anti-apoptosis and pro-inflammation help to elucidate the apoptosis-promoting and chemotherapy-reducing effects of PARP-1 inhibitors on var-

Fig. 5. PARP-1 inhibitor 4AN suppresses ATO-induced activation of NF-κB downstream effectors. The effects of the PARP-1 inhibitor 4AN on the activation of NF-κB downstream effectors, such as the anti-apoptotic Bcl-2 and pro-inflammatory iNOS, as well as the Bcl-2 partner Bax, were detected through western blot assay. The cells were treated by ATO (10 μM) in the presence or absence of 4AN (10 μM) for 24 hr. The cells treated with DMSO were used as controls. Western blot assay was performed as described in MATERIALS AND METHODS. Panels (a) and (b) represent the results of western blot assay. Panel (a) represents the western blot image. The values of iNOS, Bcl-2, and Bax were normalized to β-actin expression. Panel (b) represents the analysis of the protein expression based on the western blot image by using Quantity One software. Panel (c) represents the analysis of the Bax/Bcl-2 ratio using the data from western blot assay. Data were obtained from three independent experiments and illustrated as means ± standard deviation. “*” indicates significant differences compared with the DMSO control and the cells treated by ATO, or 4AN, or ATO+4AN. “#” denotes a significant difference compared with the cells treated by ATO and those treated by ATO+4AN.
arious anti-cancer drugs (Curtin and Szabo, 2013; Hao et al., 2013). Apoptosis is a complex process mainly regulated by the Bcl-2 family of anti- and pro-apoptotic proteins, such as Bcl-2 and Bax, respectively. The survival or death of cells through apoptosis after chemotherapy is dependent on the Bax/Bcl-2 ratio (Yu et al., 2014). Thus, the inhibitory effect of Bcl-2 by 4AN cannot sufficiently explain its apoptosis induction effect. For further investigation, we detected Bax expression. After ATO treatment, the expression of Bax up-regulated and the Bax/Bcl-2 ratio increased (Figs. 5a-5c). The PARP-1 inhibitor 4AN reduced Bcl-2 expression but did not affect Bax expression (92.97% ± 8.20% in the ATO group versus 95.53% ± 7.76% in the ATO+4AN group) (P = 0.78); as a result, the Bax/Bcl-2 ratio significantly increased (1.34% ± 0.46% in the ATO group versus 2.23% ± 0.54% in the ATO+4AN group) (P < 0.001) (Figs. 5a-5c). These findings further supported that 4AN significantly promoted ATO-induced cellular apoptosis in HepG2 cells. As Bax is not considered as a downstream gene of NF-κB, its expression may not be affected by the PARP-1 inhibitor 4AN, which targets NF-κB. Collectively, we demonstrated that PARP-1 inhibition could suppress the ATO-induced activation of NF-κB and its downstream anti-apoptosis and pro-inflammatory effectors, which may eventually lead to ATO sensitization and detoxification.

ROS-mediated DNA damage was responsible for the ATO-induced activation of PARP-1 and the resulting activation of NF-κB and its downstream effectors

We further explored the molecular basis of the involvement of PARP-1 in ATO-induced activation of NF-κB. As a DNA damage sensor protein, PARP-1 can be activated by DNA damage. The activated PARP-1 cleaves at nicotinamide adenine dinucleotide and generates long chains of poly (ADP-ribose). This process can cause chromatin relaxation and form a scaffold polymer, namely, PARP polymer, which facilitates the recruitment and assembly of DNA repair proteins (Javle and Curtin, 2011). Many anticancer agents (e.g., ionizing radiation and methyl methanesulfonate) can induce DNA damage in tumor cells and activates PARP-1-mediated DNA repair. Interestingly, recent studies demonstrated that the activated PARP-1 can physically interact with p65 through the PARP polymer; their interaction induces conformational changes in p65 and promotes p65 nuclear translocation to activate NF-κB (Hunter et al., 2012). Given that ATO exerts its anticancer effects via generation of ROS, which leads to oxidative DNA damage and apoptosis (Xia et al., 2012), and that PARP-1 is a key protein involved in base excision repair of the majority of oxidative DNA damage in mammalian cells (Javle and Curtin, 2011), we hypothesized that oxidative DNA damage induced by ATO can activate PARP-1, which then co-activates NF-κB.

To test this hypothesis, we first examined the generation of intracellular ROS and oxidative DNA damage in HepG2 cells induced by ATO, by DCFH-DA and DNA oxidative damage ELISA assays, respectively. Treatment with 10 μM ATO for 24 hr was used to trigger intracellular ROS and DNA damage. N-Acetyl-L-cysteine (NAC), a potent antioxidant, was used to alleviate ATO-induced ROS and the resulting oxidative DNA damage. Other reports and our previous studies demonstrated that 10 mM NAC is sufficient to reduce ROS level in various cell lines without affecting cell growth (Gu et al., 2015; Yang et al., 2014). Therefore, in this study, this NAC dosage was used and the results showed that it did not affect HepG2 cell growth (data not shown). Exposure to ATO significantly increased intracellular ROS and oxidative DNA damage, as demonstrated by the significantly elevated AOD and oxidized guanine (Figs. 6a-6c). This finding suggested that ATO effectively induced oxidative DNA damage in HepG2 cells via ROS generation. Moreover, NAC significantly attenuated ATO-induced ROS (0.040 ± 0.005 in the ATO group versus 0.011 ± 0.005 in the ATO+NAC group) (P < 0.001) and the oxidized guanine (212.34 ± 18.75 pg/mL in the ATO group versus 119.54 ± 14.23 pg/mL in the ATO+NAC group) (P < 0.001) (Figs. 6a-6c). Subsequently, we detected the protein expression levels of PARP-1 and NF-κB under ATO treatment in the absence and presence of NAC. Concomitant with the elevated ROS and oxidative DNA damage, the expression levels of PARP-1 and p65 nuclear translocation increased in response to ATO (Figs. 6d and 6e). This indicated that oxidative DNA damage mediated by ROS may be actively involved in PARP-1 activation and p65 nuclear translocation induced by ATO.

We further determined whether attenuation of ROS-mediated DNA damage can reverse the activation of PARP-1 and NF-κB induced by ATO. As expected, NAC alleviated ATO-induced ROS and oxidative DNA damage, thus significantly decreasing the protein expression of PARP-1 (86.03% ± 9.91% in the ATO group versus 45.03% ± 8.10% in the ATO+NAC group) (P < 0.001) and NF-κB (for nuclear 65 expression, 78.40% ± 6.82% in the ATO group versus 51.67% ± 7.35% in the ATO+NAC group (P = 0.008); for cytoplasmic 65 expression, 41.17% ± 7.64% in the ATO group versus 93.54% ± 6.54% in the ATO+NAC group (P < 0.001)) (Figs. 6a-6e). These findings supported our hypothesis that ROS-mediated oxidative DNA damage was, at least partially, responsible for...
ATO-induced activation of PARP-1 and NF-κB.

Given the critical role of NF-κB in drug resistance and toxicity, numerous inhibitors targeting the NF-κB pathway are developed to sensitize and detoxify antitumor drugs. These inhibitors include IKK complex inhibitors, such as BMS-345541, CHS-828, and parthenolide; and proteosomal inhibitors, such as bortezomib, to prevent IκB degradation (Erstad and Cusack, 2013; Olivier et al., 2006). However, the clinical application of these agents is restricted by their toxic or off-target effects caused by...
Role of NF-κB in PARP inhibitor-mediated sensitization

global NF-κB inhibition. As NF-κB plays a vital role in innate and adaptive immune responses, its long-term inhibition may increase the possibility of immune deficiency and delay bone marrow recovery after chemotherapeutic-induced apoptosis of hematopoietic stem cells (Chariot, 2009). In this regard, NF-κB needs to be maintained at an appropriate level to reserve basic immunological functions during anticancer treatment.

A recent study reported that the PARP-1 inhibitor AG-014699 inhibited the expression of NF-κB triggered by ionizing radiation-induced DNA double-strand break; the inhibited NF-κB could lead to radiosensitization in mouse embryonic fibroblast (MEF) cells (Hunter et al., 2012). However, in MEF cells treated with the immune factor TNF, AG-014699 did not affect NF-κB expression (Hunter et al., 2012). This suggested that PARP-1 inhibitors may specifically inhibit the NF-κB activation induced by DNA damage without affecting those induced by immune factors, which may help to maintain elementary immune responses during anticancer treatment. Therefore, blockade of DNA damage-activated NF-κB with PARP-1 inhibitors may represent a preferable therapeutic strategy over classical NF-κB inhibitors. This notion was partially supported by our result showing that ATO-induced oxidative DNA damage activated NF-κB in HepG2 cells in the PARP-1-dependent pathway, whereas PARP-1 inhibition with 4AN could suppress such NF-κB activation. These findings highlighted the potential of using PARP-1 inhibitors to overcome NF-κB-mediated therapeutic resistance and toxicity.

In conclusion, for the first time, we demonstrated that ATO treatment activated NF-κB and its downstream anti-apoptotic and pro-inflammatory effectors via the PARP-1-dependent pathway, which conferred HepG2 cells with ATO resistance and toxicity. PARP-1 inhibition by 4AN could suppress ATO-induced activation of the NF-κB signaling pathway, resulting in ATO sensitization and detoxification. We further explored the potential mechanism of the involvement of PARP-1 in ATO-induced activation of NF-κB. The results showed that ATO could activate PARP-1 via ROS-mediated oxidative DNA damage, and the activated PARP-1 consequently co-activated NF-κB and its downstream effectors. Our study demonstrated that the PARP-1-mediated NF-κB signaling pathway was involved in ATO resistance and toxicity in anticancer treatments. Therefore, PARP-1 inhibitors could be promising agents to mediate ATO sensitization and detoxification. Moreover, this study highlighted a novel therapeutic avenue for application of PARP inhibitors in NF-κB-mediated tumor resistance and chemotoxicity.

ACKNOWLEDGMENTS

This work was supported by grant no. 81372945 from the National Natural Science Foundation of China to Zunzhen Zhang.

Conflict of interest— The authors declare that there is no conflict of interest.

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