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Receptor Interacting Protein 3 is Required for Arsenite-Mediated Necroptosis

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Abstract

Arsenic compounds such as sodium arsenite (SA) and arsenic trioxide (ATO) are toxic to human. Primarily, we pursued to outline the cell death modes caused by arsenic compounds and to address what proteins would be responsible for arsenite-induced cytotoxicity. Both SA and ATO substantially exhibited cytotoxic activity in L929 cells. Necrostatin-1 (Nec-1) treatment significantly protected cell death mediated by arsenic compounds, suggesting that cells are committed to die in a programmed necrotic way. A geldanamycin analog DMAG destabilized receptor interacting protein 3 (RIP3) and concomitantly protected cells from SA toxicity. Using interfering RNAs, we eventually found that RIP3 was responsible for its antagonizing effects on SA. Therefore, it is proposed that arsenic compounds execute necroptotic cell death of L929 via a RIP3 dependent pathway.

Keywords: Arsenite; Heat shock protein 90; Programmed necrosis; Receptor interacting protein 3.

1. Introduction

Arsenic compounds are one of the most environmentally hazardous substances so that they are classified as human carcinogens [1, 2]. In chemistry, arsenic compounds have various oxidation states. Both pentavalent [arsenate, AS^{5+}] and trivalent [arsenite, AS^{3+}] species are most prevalent in the nature and exhibit toxicological effects on human [2].

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Particularly, arsenite acts as a double-edged sword, that is, it is associated with various cancers, including tumors of the lung, skin, bladder, and liver [3-5] while arsenic trioxide (ATO) has been widely used in the treatment of relapsed acute promyelocytic leukemia (APL) and human immunodeficiency virus (HIV) type 1 [6-8]. As can be inferred from harnessing of arsenite as an anticancer drug, arsenite has been reported to cause cell death in a caspase-dependent *manner* [9-12]. With caspase activation, generation of oxidative stress was suggested to be essential for arsenite-mediated cytotoxicity. On the contrary, there have been the report that arsenic trioxide (ATO) is effectively used in treatment of malignancies via a caspase-independent pathway. Specifically, ATO induces autophagy in T-lymphocytic leukemia and myelodysplastic syndrome, and notably in malignant gliomas, which show resistance to various commonly used therapies [13-16]. Also, it is involved in triggering a caspase-independent necrotic cell death via the mitochondrial pathway [17, 18]. When it comes to cell death, necrosis and apoptosis are exclusively distinctive in the aspects of morphology and its underlying molecular events [19, 20]. Meanwhile, a specialized necrosis coined "programmed necrosis or necroptosis" has been generally regarded as an alternative cell death mode activated under the specific condition when tumor necrosis factor alpha (TNFα)-mediated apoptotic machinery is defective [21]. Besides TNFα, it has been possibly suggested that a few chemicals and heavy metals can induce necroptosis-like cell death, distinct from apoptosis [22]. Similar to TNFα-mediated necroptosis, chemicals- or heavy metal-induced cell death is reversed by necrostatin-1 (Nec-1), a specific inhibitor receptor interacting protein 1 (RIP1) [23-25] . Once TNFα receptor (TNFR) ligated, RIP1 functions as a scaffolding protein of TNFα signaling, and transmits death signals to downstream effectors by forming complex with RIP3. Therefore, RIP1 and RIP3 are proposed to be crucial proteins that can determine cell death in favor of necroptosis when cells are subjected to stresses [26-28]. Here, we hypothesized that a chemical but not TNFα could also induce necroptosis through RIP1 or RIP3. Furthermore, we sought to delineate the underlying mechanisms by which arsenite could induce cell death via non-apoptotic pathway. To this end, some chemicals were preliminary tested if those chemicals would mediate necroptosis-like cell death. In particular, sodium arsenite (SA) could effectively promote cell death in L929, a specialized cell line for necroptosis. L929 cells used in this research have been known as a model system of necroptosis that can be induced upon TNFα stimulation since they are defective in caspase activation. The cell proliferation assay (MTS) and flow cytometric (FACS) data demonstrated that both sodium arsenite and ATO were cytotoxic at a 20 μM concentration, ATO being slightly more potent than SA. SA-mediated killing effects were significantly reversed by Nec-1 but not zVAD, a pan-caspase inhibitor. Furthermore, RNA interference study revealed that RIP3 but not RIP1 was responsible for SA-mediated necroptotic cell death. Taken together, we suggest that SA induces necroptosis-like cell death via a RIP3-dependent route. This study will provide mechanistic basis for understanding SA-triggered signaling pathways leading to cell death, and support its clinical feasibility to fight against drug-resistant cancers that can evade apoptosis surveillance.

2. Materials and Methods

2.1. Reagents

Sodium arsenite (SA) and arsenic trioxide (ATO) were obtained from Wako (Richmond, VA, USA). zVAD-fmk (zVAD), necrostatin-1 (Nec-1), *N*-acetylcystein (NAC), butylated hydroxyanisole (BHA), propidium iodide (PI) and 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA) were available from Sigma-Aldrich (St. Louis, MO,

USA). The 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (DMAG), a geldanamycin (GA) derivative, was kindly given from **Ulsan National Institute of Science and Technology** (UNIST, Ulsan, South Korea). Anti-RIP1 and anti-RIP3 were from BD Pharmingen (San Diego, CA, USA) and ProSci (Poway, CA, USA), respectively. Cell proliferation assay kit (MTS reagent, a tetrazolium salt) was bought from Promega (Madison, WI, USA). HiperFect as a transfection reagent of siRNAs was purchased from Qiagen (Valencia, CA, USA). FITC-annexin V (AnxV) detection kit was bought from BD Biosciences (Franklin Lakes, NJ, USA). Other chemical reagents used were of analytical grade.

2.2. Cell line and cell culture

L929, a mouse fibrosarcoma cell line (ATCC CCL-1) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were grown in high-glucose Dulbecco's modified Eagle's (DME) medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS), and allowed to incubate at 37°C in a 5% CO_2 incubator.

2.3. Dose responses of cells to arsenic compounds and effects of Nec-1 on arsenic chemicals-induced cytotoxicity

L929 cells were cultured in a 96-well plate at a density of 1 x 10^4 cells/well. Cells were exposed to increasing doses of SA and ATO (10-50 μM). Furthermore, cells were treated with SA (20 μM) and ATO (10 μM) at a fixed concentration in the presence or absence of 10 μM Nec-1 for 24 h. To measure the cell viability, MTS assay was performed according to the manufacturer's protocol.

2.4. Effects of DMAG on SA-mediated cell toxicity

Primarily, cellular levels of two necroptosis regulators RIP1 and RIP3 after DMAG pretreatment were examined by immunoblot. Cells were pretreated with DMAG (1 and 5 μM) for 15h and then were lysed in M-PER reagent (PIERCE, Rockford, IL) for 20 min on ice. The resulting lysates were spun in a microcentrifuge for 20 min at 4°C, and then the supernatants were taken. Aliquots of the cell lysates (20 μg) were run on 10% SDS–polyacrylamide gels (SDS-PAGE), and Western blotting was performed with antibodies against RIP1, RIP3 and β-actin. The interest proteins were visualized by enhanced chemiluminescence (ECL) in accordance with the manufacturer's instructions. To examine the effects of DMAG pretreatment on SA- or ATO-caused cytotoxicity, cells were pretreated with 1 μM DMAG 15 h and then exposed to SA or ATO at 20 μM and 10 μM, respectively. Furthermore, to verify the role of Nec-1 on arsenic compounds after DMAG treatment, DMAG-pretreated cells were left treated with SA or ATO together with 10 μM Nec-1. Following 24 h incubation, cell viability was determined by MTS.

2.5. RIP1- or RIP3-specific siRNA design, synthesis and transfection

Interference RNAs targeting RIP1 or RIP3 were specifically designed as described below. In brief, siRNA sequences were deduced from uploading cDNA sequences into the query form of the Integrated DNA

Technologies (IDT) Web server. The resulting siRNAs were synthesized by Bioneer (Daejeon, South Korea). After repetitive testing of siRNAs prepared, a specific siRNA that can downregulate RIP1 or RIP3 expression was finally chosen for subsequent experiments. The siRNA sequences for RIP1 and RIP3 were listed in the previous article [26]. For reference, TNFα-related apoptosis-inducing ligand receptor 4 (TR4) siRNA was used unless otherwise stated. Transfection of siRNAs into cells was carried out as previously described [26]. Briefly, either RIP1 or RIP3 siRNA (100 nM) selected was transfected into 0.25 ml culture media of L929 cells using HiperFect (6 μl) as a transfection agent according to the instruction manual provided by the supplier. On the next day, the cells were exposed to arsenite or arsenite plus Nec-1. Cytotoxicity was determined by MTS assay 24 h after exposure to arsenic compounds, and expressed as % viability of treated group relative to the untreated control.

2.6. Flow cytometric analyses

L929 cells were plated at 2 x 10^5 cells/each well of the tissue culture plates. For FACS analyses (FACSVerse instrument, BD Bioscience, CA), cells were treated with various combinations of test compounds under examination for 24 h, and then stained with AnxV and PI. For statistical significance, at least 10,000 cellular events were acquired per sample to get the % cell death quantified.

2.7. Intracellular ROS detection

Cells were treated with 5 μM cell-permeant 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA) fluorescent probe for 1 h, and then dissociated with trypsin. Flow cytometer is employed to detect intracellular ROS production in collected cells. A total of 10,000 events were recorded for the fluorescence of DCFDA on FL-1 channel (520 nm).

2.8. Statistics

The results obtained were expressed as mean ± standard deviation from at least three independent experiments. The significance was set at $p < 0.05$ for each analysis using student's *t*-test.

3. Results

3.1. Dose responses of L929 to arsenic compounds for their cytotoxicity, and protection from arsenite-mediated cytotoxicity by Nec-1

Of most common trivalent arsenic compounds, SA and ATO were tested in L929 cells to plot dose-response curves for cytotoxicity. As indicated in MTS assay of Fig. 1A, SA was toxic over 10~20 μM concentrations whereas ATO killed cells more drastically even at a lower concentration of 10 μM, and thereafter, both SA and ATO kept cells viable at a 50% level. As an effort to roughly outline SA-mediated cell death, a RIP1 inhibitor Nec-1, which regulates programmed necrosis, was employed (Fig. 1B).

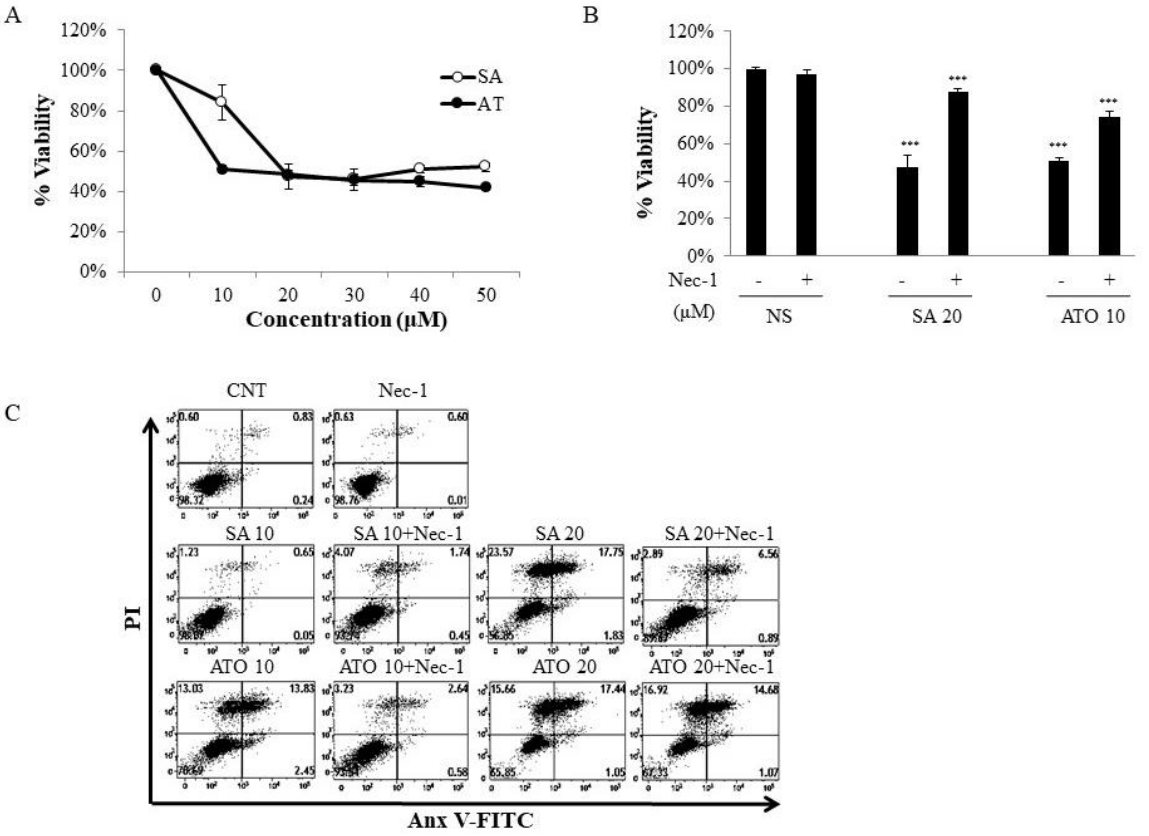

Figure 1: Dose responses of L929 to arsenic compounds, and protective effects of Nec-1 against arsenic compounds. (A) To plot dose-response curves of cells to sodium arsenite (SA) and arsenite trioxide (ATO), cells were exposed to various concentrations of arsenic chemicals, ranging from 10 to 50 μM for 24 h. (B) Also, a RIP1 specific inhibitor Nec-1 was employed to delineate cell death mode caused by arsenite. Cells were treated with either SA (20 μM) or AT (10 μM) in the presence or absence of Nec-1. Cell toxicity was measured by MTS assay according to the manufacturer's protocol and expressed as % viability relative to control group. (C) Flow cytometric analyses of cells exposed to arsenite alone or arsenite plus Nec-1. Cells grown in a 12 well-plate were treated with SA or ATO (10 and 20 μM) in the absence or presence of 10 μM Nec-1 for 24 h, and then stained with AnxV and PI. Each cell in the live or dead cell populations was analyzed by FACS. Statistically significance: ***$p<0.005$ against group in the absence of Nec-1.

Cells treated with either 20 μM SA or 10 μM ATO was significantly protected in the presence of Nec-1, although cells exposed to ATO was less rescued than those subjected to SA exposure by a necroptosis inhibitor. To further examine cell death profiles caused by SA, FACS analyses were carried out after cells were stained with PI and AnxV-FITC (Fig. 1C). In line with MTS results, SA at a lower concentration (10 μM) did not cause cell death substantially. However, nearly 50% of total cells were dead when treated with 20 μM SA, revealing that one half of dead cell population was double positive for PI and AnxV and the other half was single positive for PI. When compared with SA, ATO caused considerably cell death at 10 μM concentration, but did not seem to exhibit a linear dose response over 10~20 μM as anticipated in MTS results. Moreover, Nec-1 treatment effectively reduced the dead cell populations consisting of PI-single positive and PI/AnxV-double positive cells

which were induced by SA. Unlike SA, though, development of cell death caused by 20 μM ATO was not reversed by Nec-1 treatment.

3.2. The protective effects of DMAG on SA-caused cytotoxicity in L929 cells

Cytotoxic responses of L929 cells to SA were further monitored following treatment of DMAG, an Hsp90 inhibitor. Primarily, expression levels of key necroptosis regulators RIP1 and RIP3 were investigated when Hsp90 was functionally inhibited by DMAG. Immunoblot analysis revealed that DMAG treatment noticeably led to degradation of RIP1 and RIP3 in a dose dependent manner (Fig. 2A).

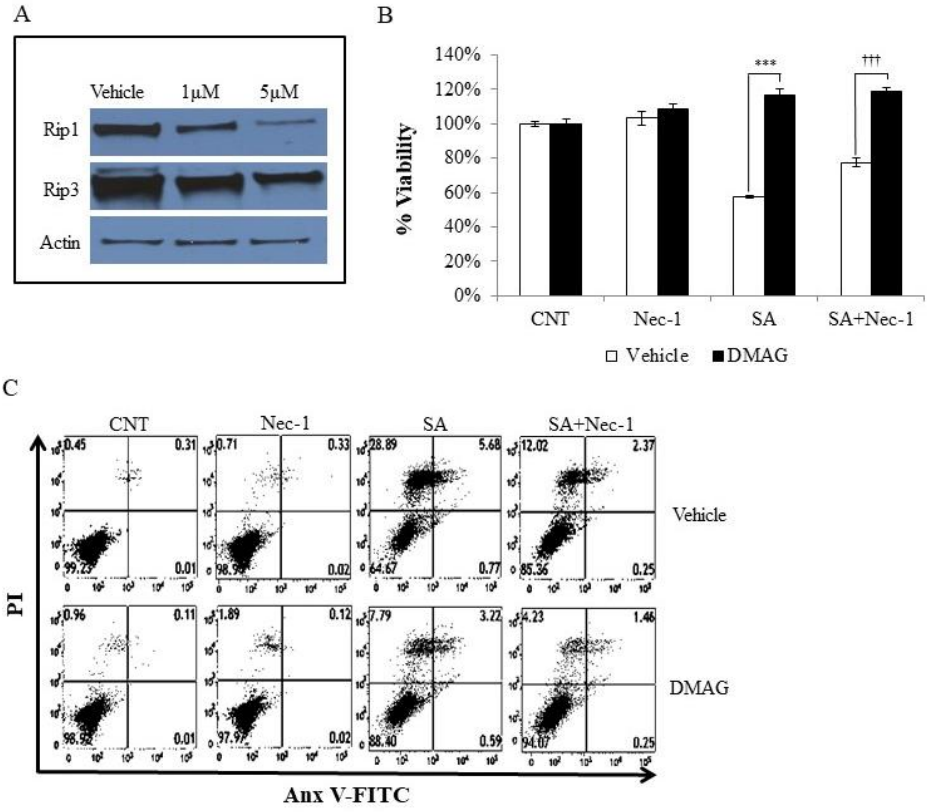

Figure 2: The effects of DMAG on SA-mediated cytotoxicity in L929 cells. (A) RIP1 and RIP3 expression in L929 cells which were treated with DMAG. Cells were treated with 1 and 5 μM DMAG for 15 h, and then harvested cells were lysed to run the resulting lysates onto SDS-PAGE. Transferred membranes were immunoblotted with antibody against RIP1 or RIP3. (B) Cytotoxic responses of DMAG-pretreated cells to SA. L929 cells were pretreated with 1 μM DMAG and then exposed to 20 μM SA for 24 h. Nec-1 (10 μM) was also tested to monitor whether it could still be effective in protecting SA-mediated cell death following DMAG pretreatment. Cell viability was evaluated by MTS assay. (C) Flow cytometric analyses of L929, which were pretreated with DMAG or vehicle, subjected to SA exposure. L929 cells plated into a 12 well plate were pretreated with DMAG for 15 h and then were exposed to 20 μM SA for 24 h. The harvested cells were stained with AnxV and PI and then cell survival/death profiles were determined by flow cytometry. Shown are the representative flow cytometric dot plots of three independent data. ***$p<0.005$ versus vehicle group.

RIP1 proteins disappeared more rapidly than RIP3 upon DMAG exposure. Moreover, MTS and FACS data showed that DMAG pretreatment protected effectively cells from SA-mediated cell death (Fig. 2B and 2C). Protective effects of DMAG against SA-induced cytotoxicity were more manifested than those of Nec-1. MTS data revealed that cells pretreated with DMAG were refractory to SA exposure. Furthermore, flow cytometry reinforced that most PI-positive populations in SA-treated group were significantly reduced by DMAG, indicating that protective effects of DMAG are fairly superior or equivalent to Nec-1. As expected in MTS results, Nec-1 itself exhibited the same antagonizing effects on cells exposed to SA. Cells treated with DMAG and Nec-1 was protected from SA toxicity in a similar level to those with DMAG alone.

3.3. Role of RIP1 or RIP3 on SA-executed cell death

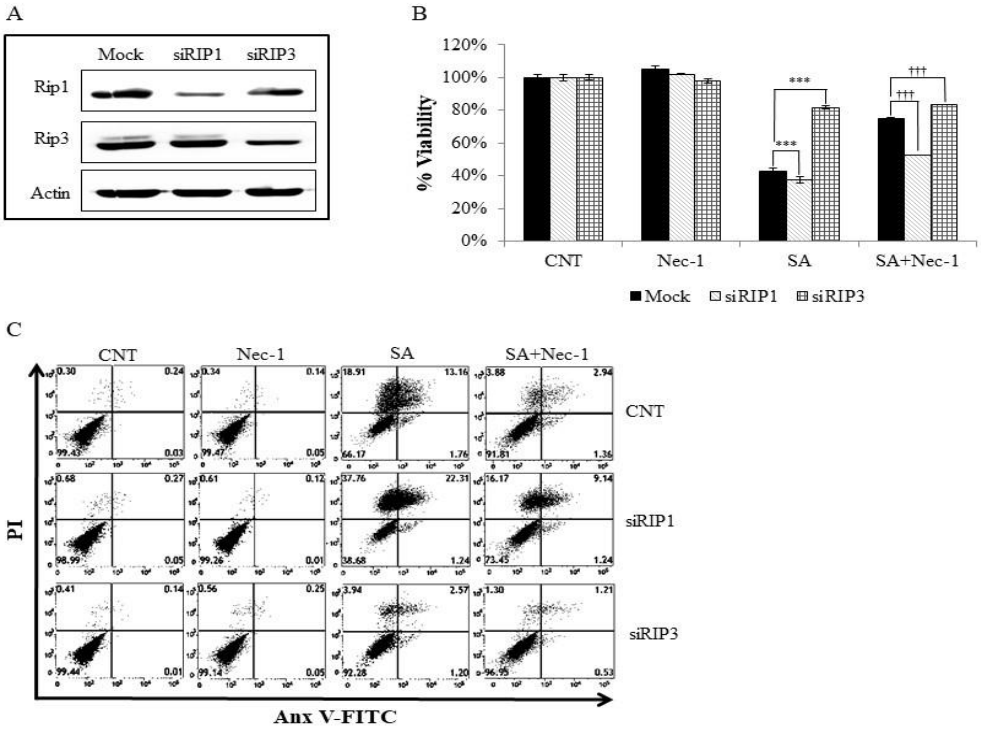

Figure 3: The differential effects of RIP1 or RIP3 knockdown on SA-mediated cell death. (A) Expression levels of RIP1 and RIP3 following a RNA interference targeting RIP1 or RIP3. Silencing protocols were carried out as described in Materials and Methods. To check the outcomes of RNA interferences, L929 cell lysates were prepared and subjected to SDS-PAGE for immunoblotting with antibodies against RIP1 or RIP3. (B) Effects of RIP1 or RIP3 knock-down on SA-mediated cytotoxic activity. Following siRNA transfection, the cells were subjected to stimuli such as arsenite or arsenite plus Nec-1. Cytotoxicity in L929 cells was determined by MTS assay 24 h after exposure to arsenic compounds, and expressed as % viability of treated group relative to the untreated control. (C) Flow cytometric analyses of L929, which were knocked-down with RIP1 or RIP3 siRNAs, subjected to SA exposure. RIP1 or RIP3 knocked-down L929 cells were stimulated with 20 μM SA for 24 h. The subsequent cells were stained with AnxV and PI, quadrant profiles of which were determined by flow cytometry. Shown are representative data out of at least three independent measurements. ***$p<0.005$ against mock group.

To address which death-specific molecules could be involved in arsenic compounds-mediated cytotoxicity, expression levels of RIP1 or RIP3 were reduced by using RNA interferences (Fig. 3). Western blot demonstrated that down-regulation of either RIP1 or RIP3 was well verified in cells transfected with siRNA specific to it (Fig. 3A). In this experiment, transient down-regulation of RIP1 or RIP3 itself did not affect cell viability considerably (data not shown). Responses of cells to SA were investigated following the knock-down of either RIP1 or RIP3 gene (Fig. 3B). As shown in MTS data, interference of RIP3, but not of RIP1 protected significantly cells from SA-mediated toxicity. Interestingly, Nec-1 failed to relieve the toxicity of SA in RIP1 knocked-down cells. For more details, FACS analyses were carried out with cells subjected to the same conditions as above (Fig. 3C). It was of note that RIP1 silencing rendered cells sensitive to SA as compared with mock control group. Roughly, PI-positive cells in RIP1-silenced group were increased as double as those in mock group. By contrast, RIP3 silencing protected cells from SA toxicity. Unlike RIP1 knocked-down cells, intriguingly, application of Nec-1 to cells transfected with mock or siRIP3 effectively rescued cells from SA-mediated toxicity.

3.4. Intracellular ROS production and antioxidants' effects in SA-treated L929 cells

Cell death modalities and intracellular ROS in SA-treated L929 cells were determined by flow cytometer (Fig. 4A and 4B). The flow cytometric analyses demonstrated that SA-mediated cell death was significantly rescued by both ROS scavengers, NAC and BHA (Fig. 4A). Specifically, SA treatment caused cells to be in the upper right quadrant being double-positive for AnxV and PI. $AnxV^+/PI^+$ double positive events (40 %) in cells exposed to SA were reduced to as low as 2~3 % by either NAC or BHA. Consistent with AnxV/PI data, ROS-positive live population was considerably increased when L929 cells were subjected to SA exposure (Fig. 4B). After treatment of cells with either SA alone or SA in combination with ROS scavengers, changes in ROS production were analyzed in flow cytometry (Fig. 4B). ROS production in cells treated with SA was remarkable, as indicated by an increase (30 %) in events of P1 region relative to non-stimulated group (NS). Not only NAC but also BHA obviously reversed SA-induced ROS generation although the scavenging effect of NAC appeared to be more effective than that of BHA.

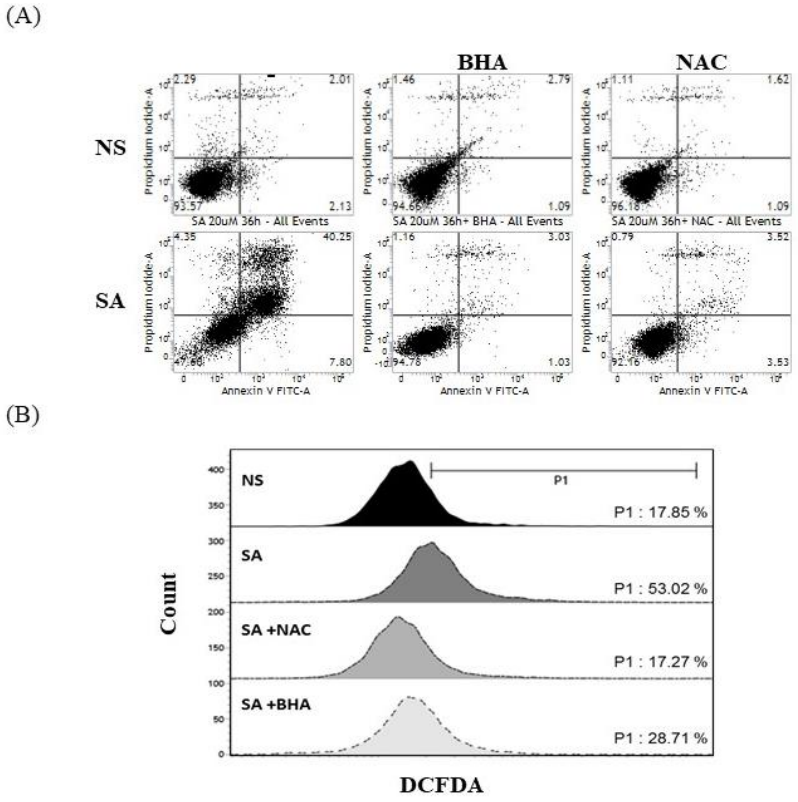

Figure 4: The involvement of ROS in SA-induced cytotoxicity. L929 cells were preincubated with 5 mM NAC or 0.1 mM BHA for 1 h, and then exposed to 20 μM SA for 24 h. Each treated group was stained with AnxV and PI for analyzing cell death modality (A). Another set of experiments was undertaken for the same treated group as above, and then cells was stained with 5 μM DCFDA for 1hr and then subjected to flow cytometric analyses for intracellular ROS detection (B). The same numbers of events, gated from SSC/FSC plot of treated group, were represented in histogram for DCF fluorescence intensity. Percentage of a population within P1 region showing events with high fluorescence intensities was determined to compare ROS levels generated between treated groups. All data are representative of three independent flow cytometric analyses.

4. Discussion

Arsenite plays pleiotropic effects on a variety of biological events, including inflammation, cell survival and death. Specifically, arsenite is proposed to invoke vascular inflammation and further to develop pathology of vascular disease by enhancing the TNFα-induced VCAM-1 expression via regulation of AP-1 and NF-κB activities [29]. In addition, it has recently been revealed that embryonic stem cells exposed to SA do not make self-renewal and undergo apoptotic death via suppression of AKT and Stat3 activation [30]. In this article, it has been demonstrate that cell cycle is arrested on G2/M and apoptosis is later executed by the mitochondrial pathway. Furthermore, ATO has been reported to have differential effects on death modes of cells derived from various tissues [31-33]. Specifically, HeLa, calf pulmonary artery endothelial cells (CPAEC) and human umbilical vein endothelial cells (HUVEC) undergo apoptotic cell death when treated with ATO. However, there

have been still contradictory reports that arsenic compounds cause cell death in a caspase-independent manner. ATO triggers caspase-independent necrotic cell death via Bcl-$_{XL}$-sensitive mitochondrial pathway [17]. Moreover, blockade of glutathione synthase by buthionine sulfoximine (BSO) augments ATO-mediated necrosis, reasoning that ROS are substantially involved in necrosis-like cell death. Initially, an alternative cell death mode called programmed necrosis or necroptosis has been introduced to describe the backup cell death unmasked when the default cell death (apoptosis) is defective upon TNFα stimulation. Besides an inflammatory cytokine TNFα, however, a number of chemicals or heavy metals have been reported to provoke necroptosis-like cell death. Shikonin and alkylating agent cause cell death with non-apoptotic features by various criteria [34, 35]. Notably, severe DNA damage by alkylating agent is suggested to activate PARP-1 that overuses NAD^+ as a substrate, eventually causing energy crisis and necrosis [36]. Whatever it may be TNFα or other necrotic death inducers, application of Nec-1 effectively block cells from undergoing necroptotic cell death. Accordingly, it is tempting to speculate that chemicals or heavy metals, like TNFα-induced necroptosis, may also require a series of its associated proteins for mediating necroptotic cell death. Meanwhile, DMAG is an inhibitor of Hsp90, which is required for stability and activity of a variety of client proteins. Among the interacting proteins of Hsp90, RIP1 has been already known as a client protein, and RIP3 has recently been suggested to be a putative client candidate of it [22, 37]. In fact, the functional disruption of Hsp90 results in degradation of death domain kinase RIP, and subsequent suppression of TNFα-induced NFκB activation, proposing that RIP1 is necessary for TNFα/NFκB signal transduction [37, 38]. As a result, RIP1 depletion sensitizes cells to TNFα by switching from necrosis to apoptosis, indicating that RIP1 can function as an apoptosis suppressor in L929 cells [39]. Under the condition that RIP1 and RIP3 were destabilized by an Hsp90 inhibitor, in our result, SA-mediated cytotoxic activities were considerably ameliorated compared with vehicle group, reckoning that either RIP1 or RIP3 contributes to reduction of cellular damage caused by SA. However, it could not be ruled out that other client proteins than RIPs might be responsible for the protective effects of DMAG. To address what necroptosis-regulating proteins are involved in the refractory response of DMAG-treated cells to SA exposure, RNA interferences targeting RIP1 or RIP3 were primarily employed. Intriguingly, RIP3 knockdown effectively protected cells from SA-induced cytotoxicity whereas RIP1 silencing rendered cells more sensitive to SA. It can be suggested that RIP3 but not RIP1 is prerequisite for SA-mediated cell death. We have already demonstrated that either TNFα or zVAD causes cell death in a RIP3- or RIP1-dependent pathway, respectively. Therefore, it might be plausible that SA could transmit directly its death signal to RIP3 via an unidentified route. Otherwise, SA could provoke TNFα secretion from L929 cells, which are subsequently liable to undergo necroptotic cell death via death receptor. Moreover, exposure of RIP1 knocked-down L929 cells to SA was not rescued by Nec-1 treatment, suggesting that adequate expression of RIP1 is essential for protective efficacy of Nec-1 against necroptosis. Nec-1 has been initially developed as a necroptosis inhibitor targeting specifically RIP1. However, it has been proposed that Nec-1 can have off-target effects or may target other molecules than RIP1. According to our latest results, Nec-1 protects cells from either TNFα or zVAD, which is proposed to transmit death signal through a RIP3- or a RIP1-dependent route, respectively [22]. Moreover, Nec-1 has been reported to exerts RIP1-dependent and –independent effects in the process of necroptosis and T cell activation [40]. Although there remain controversies regarding authentic molecular target of Nec-1, it is still evident that protective effects of Nec-1 depends on RIP1 expression.

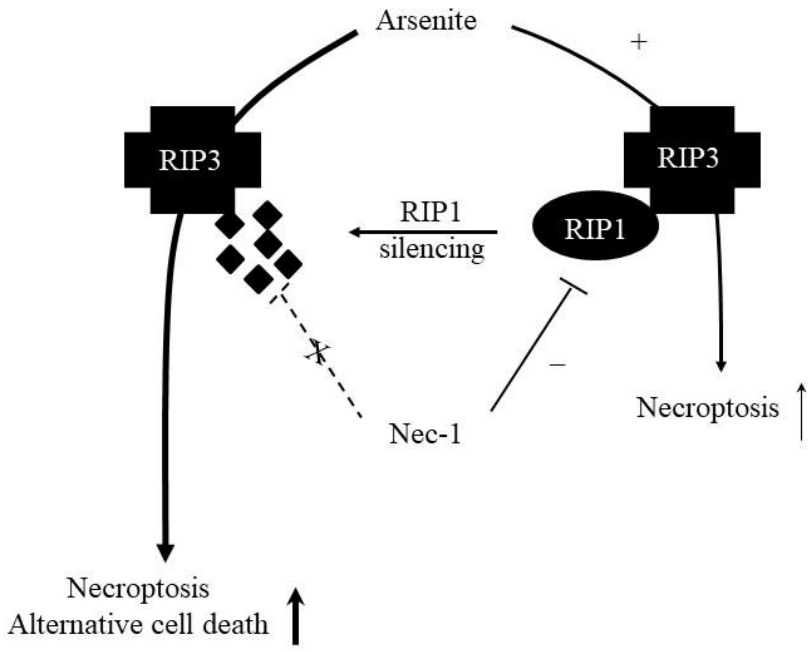

Figure 5: A proposed schematic model for SA-mediated cell death in L929 cells. Both RIP1 and RIP3, two well-known key regulators of TNFα-mediated necroptosis, are also required for SA-induced cytotoxicity. RIP3 in combination with RIP1 can execute SA-mediated necroptosis, which can be reversed by a RIP1-specific inhibitor Nec-1. RIP1 knockdown via RNA interference enhances cytotoxic response to SA considerably, promoting necroptotic cell death. However, even Nec-1 treatment did not protect L929 with low levels of RIP1 from SA toxicity. Symbols – and + indicate protection and promotion of necroptosis, respectively, and a symbol X represents that Nec-1 is not effective on SA-induced necroptotic cell damage at the low levels of RIP1.

Among several possible mechanisms for SA cytotoxicity, ROS have been suggested to be one of decisive mediators to execute cell death. Low doses of arsenite induce ROS production and ROS-associated mitochondrial membrane depolarization [41, 42]. During SA-mediated tumor promotion, hydrogen peroxide is a key player of signaling pathway for activation of $p70^{s6k}$ and extracellular signal-regulated kinase [43, 44]. As with being consistent with SA-caused time-dependent viability loss (MTS data), in our results, SA induced ROS production in a time dependent manner. Both a potent antioxidant NAC and a lipophilic ROS scavenger BHA blocked ROS generation from SA-treated cells, protecting cells from SA-mediated damage. This result was different from previous data showing the contradictory effects of two ROS scavengers against TNFα-induced ROS [21, 22]. It has been reported that BHA but not NAC neutralizes TNFα-generated ROS production with effective protection of TNFα-induced necroptosis. It suggests that intracellular sources and reactive intermediates of ROS produced by TNFα can be different from those produced by SA. Although both TNFα and SA require RIP3 to promote caspase-independent cell death, intracellular reactive intermediates including ROS can be differently induced in a death content-specific manner. As summarized in Fig. 5, SA induces necroptosis-like cell death in a RIP3 dependent manner. Expression levels of another key necroptotic protein RIP1 affect

SA-induced cell death significantly. In fact, knockdown of RIP1 by RNA interference makes L929 cells more sensitive to TNFα than normal levels of RIP1 do. Also, a RIP1 specific inhibitor Nec-1 effectively reverses SA-mediated toxicity in the presence of RIP1 while it does not in RIP1 knocked-down cells. This study suggests that expression of RIP1 can regulate positively or negatively SA-mediated necroptosis. For instances, basal levels of RIP1 can to some extent suppress RIP3-mediated necroptosis, but depletion of cellular RIP1 by silencing can render cells more sensitive to SA.

5. Conclusion

In conclusion, we propose that SA mediates necroptotic cell death of L929 cells via a RIP3-dependent pathway. Extensive efforts will be further carried out to disclose its underlying mechanisms and identification of proteins relevant to arsenite-mediated cell death. These consequences will provide perspectives on the feasibility of SA's clinical use or on chemo-preventive strategy against SA.

6. Conflict of Interest

The author declares no directly or indirectly conflict of interest.

Acknowledgements

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Author resposnses to reviwer's Comments

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Accept As Is:	
Requires Minor Corrections:	X
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A manuscript has been suited according to the template. Also, As reviewers recommended, purpose and discussion of this study were revised to make them clear. First of all, the hypothesis and purpose of this study were additionally refined in the introduction part. Next, additional discussion on the results was done to make readers clarify at the end of discussion part.