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Glutathione selectively inhibits Doxorubicin induced phosphorylation of p53Ser^{15}, caspase dependent ceramide production and apoptosis in human leukemic cells

Faisal Thayyullathil, Shahanas Chathoth, Jaleel Kizhakkayil, Alaa Galadari, Abdulkader Hago, Mahendra Patel, Sehamuddin Galadari *

Cell Signaling Laboratory, Department of Biochemistry, Faculty of Medicine and Health Sciences, UAE University, P.O. Box 17666, Al Ain, United Arab Emirates

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ABSTRACT

Glutathione (GSH) is the most abundant non-protein antioxidant in mammalian cells. It has been implicated in playing an important role in different signal transduction pathways, and its depletion is an early hallmark in the progression of apoptosis in response to a number of proapoptotic stimuli. We have selectively investigated the role of GSH in cytotoxic response of Jurkat and Molt-4 human leukemic cells to the anti-cancer drug Doxorubicin. In this study, we have shown that extracellular supplementation of GSH to human leukemic cells renders them a resistant phenotype to Doxorubicin treatment. Glutathione pretreatment inhibits Doxorubicin-induced p53Ser^{15} phosphorylation, caspase dependent ceramide (Cer) generation, Poly (ADP-ribose) polymerase (PARP) cleavage, and DNA fragmentation. Taken together, these results indicate that the major cellular antioxidant GSH influences the chemotherapeutic efficacy of Doxorubicin towards human leukemic cells.

1. Introduction

Glutathione (GSH) is the most abundant non-protein thiol in mammalian cells, which acts as a major antioxidant within cells by maintaining a tight control on cellular redox status. Intracellular GSH depletion is an early hallmark in the progression of apoptosis in response to various cellular stresses [1]. It has been previously reported that depletion of GSH leads to ceramide (Cer) production, and apoptosis in MCF-7 cells [2]. Glutathione depletion during apoptosis, induced by cytotoxic agents, has been reported to mediate GSH oxidation to GSSG through the action reactive oxygen species (ROS). Previously, from our laboratory, we have shown that curcumin induces ROS-dependant depletion of GSH, and initiation of caspase dependant and caspase independent apoptosis in L929 cells [3]. High intracellular GSH has been associated with an apoptotic resistant phenotype in several models of apoptosis [4].

The tumor suppressor protein p53 is a potent transcription factor which is activated in response to DNA-damaging agents; including ultraviolet radiation (UVC) [5], and certain genotoxic chemicals [6,7]. The stability and activation of this protein is controlled by posttranslational modifications such as phosphorylation and acetylation. Several phosphorylation sites have been identified on the amino terminal of p53 [8]. However, the most well-studied phosphorylation site is Ser^{15}, which is known to be essential for the transactivation of p53 [9].

Ceramide has been suggested to be a "tumor suppressor lipid" and is able to exert potent growth suppressive effect in a variety of cell types [10]. A diverse array of stresses, including TNF-α, Fas ligation, irradiation, heat shock, and anti-cancer drugs have been reported to increase intracellular Cer and induction of apoptosis [11–15]. Caspases, a family of cysteine-dependent aspartate directed proteases, play a critical role in the initiation and execution of apoptosis. Caspase-dependent Cer generation has been previously proposed in several apoptosis models [16–19]. Moreover, it has been reported that cell permeable Cer induces the cleavage and activation of caspase-3 [15]. Therefore, it has been proposed that caspase-3 can act both upstream and downstream of Cer generation [15–18]. The relationship between caspase activation and Cer production in apoptosis signalling is still not well understood. Previously, caspase regulated Cer generation has been proposed in Fas-induced apoptosis in human leukemic cells [20].

Doxorubicin is a major anti-cancer drug widely used in the treatment of acute myeloid leukemia, malignant lymphoma, and solid tumor treatment [21]. It has been proposed that apoptosis induced by Doxorubicin was mediated by Cer, either, through

Abbreviations: Cer, D-*erythro*-ceramide; PBS, phosphate buffered saline; PARP, poly (ADP-ribose) polymerase; PAGE, polyacrylamide gel electrophoresis; z-VAD-fmk, N-benzoyloxycarbonyl–Val–Ala–Asp fluoromethylketone; Dox, Doxorubicin; GSH, glutathione; NAC, N-acetyl cysteine.

* Corresponding author. Fax: +971 37672033.
E-mail address: sehamuddin@uaeu.ac.ae (S. Galadari).

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synthesized *de novo* [22], or through Sphingomyelin hydrolysis [23]. Moreover, it has been reported that Doxorubicin can also induce caspase dependent apoptosis in Jurkat cells [24]. In the present study, we demonstrate that blockage of Doxorubicin-induced apoptosis by GSH is through inhibition of p53 phosphorylation at Ser^{15} residue, caspase dependant Cer generation, and DNA fragmentation.

2. Materials and methods

2.1. Leukemic cell lines, cell culture conditions drug treatment

The cell lines Jurkat and Molt-4 (ATCC, Rockville, MD, USA) were grown in RPMI 1640 containing GlutaMAX medium supplemented with 10% (V/V) heat inactivated fetal bovine serum (FBS). All cell lines were grown without antibiotics in an incubator containing humidified atmosphere of 95% air and 5%CO_2 at 37 °C. Doxorubicin (Sigma Chemical Co. St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, and was stored in a dark colored bottle at −20 °C. The stock was diluted to the required concentration with DMSO when needed. Prior to Doxorubicin treatment cells were grown to about 80% confluence, and then exposed to Doxorubicin at different concentrations (0–1 μM), and for a different period of time (0–24 h). Cells grown in a medium containing an equivalent amount of DMSO served as control. z-VAD-fmk was from Alexis (San Diego, CA, USA). GSH and NAC (Sigma Chemical Co. St. Louis, MO, USA).

2.2. Cell viability Assay

Cell viability assay were carried out as described elsewhere with slight modifications [25]. Cells were grown in 96 well microtiter plates (10,000 cells/well), and they were incubated for 24 h with or without different concentration of Doxorubicin. At the required time point 100 μl media were removed and 25 μl of MTT (5 mg/ml) was added to each well. The plates were incubated for a further 4 h at 37 °C. After incubation the plates were centrifuged at 1500 rpm for 5 min and the media were removed from all the wells. The formazan crystals were then solubilized in a 200 μl of DMSO. The colored solution was quantified at 570 nm by using 96 well plate reader (Perkin Elmer spectrofluorometer, Victor 3×). The viability was expressed as a percentage over control.

2.3. Protein lysate preparation and western blot analysis

Cells were washed twice with phosphate buffered saline (PBS), and lysed in a RIPA lysis buffer [50 mM Tris HCl (pH 7.4), 1% NP-40, 40 mM NaF, 10 mM NaCl, 10 mM Na_3VO_4, 1 mM phenylmethylsufonyl fluoride (PMSF), and 10 mM dithiothreitol (DTT) and EDTA-free protease inhibitor tablets per 20 ml buffer]. The cell lysates were centrifuged at 14,000 rpm for 15 min. Total protein, determined by Bio-Rad protein assay, were mixed with 6× loading buffer and boiled at 100 °C for 3 min. SDS–PAGE and Western blot analysis were carried out as described previously [26]. The following antibodies were used: Anti-actin, p53, and donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-PARP and anti-phospho $p53Ser^{473}$ were from Cell Signaling Technology. Anti-rabbit IgG and anti-mouse IgG were purchased from Sigma chemicals Co (St. Louis, MO, USA).

2.4. Intracellular Cer measurement

Intracellular Cer measured as described previously with little modification [27]. After treatment cells were washed in PBS and lysed 50 mM Tris (pH-7.4) containing 0.4% IGEPAL CA 630 by freeze and thaw method. The final concentration of IGEPAL CA 630 in the assay was 0.2%. The lysate were heat at 70 °C for 5 min in a water bath and centrifuged at 12,000 rpm for 10 min at 4 °C. The reaction was started by adding 10 μl of supernatant in the tube containing 20 ng recombinant human neutral ceramidase enzymes (10 μl) for 1 h at 37 °C. The reaction was stopped by adding 55 μl of stopping buffer (1:9, 0.07 M potassium hydrogen phosphate buffer: methanol). The released SPH was derivatized with o-phthaladehyde (OPA) reagent. After stopping the reaction add 25 μl of freshly prepared OPA reagent (12.5 mg OPA dissolved in 250 μl ethanol and 12.5 μl β-mercaptoethanol, and made up to 12.5 ml with 3% (w/v) boric acid) was added. The mixture was allowed to stand for 30 min. An aliquot of 25 μl was injected in the HPLC. HPLC analysis was done using *Waters 1525* binary pump system. Waters XTerra C18 column (5 μm, 3 mm × 250 mm) was equilibrated with a mobile phase (20% methanol, 80% 1:9, 0.07 M potassium hydrogen phosphate buffer: methanol) at a flow rate of 0.5 ml/min. The fluorescence detector (*Waters 2475*) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

2.5. DNA fragmentation analysis

Apoptotic DNA fragments were isolated from the apoptotic cells as described previously [3]. Cells were grown at a density of 2×10^6 cells per plate and incubated with various concentrations of Doxorubicin. Cells were then washed with PBS and incubated with 200 μl of lysis buffer (50 mM Tris–HCl (pH 7.5), 3% non-ionic detergent IGPAL CA-630 [(Octylphenoxy) polyethoxyethanol] and 20 mM EDTA) for 10 min. The samples were centrifuged at 1000 × *g* for 5 min in order to collect the supernatant which contain apoptotic DNA fragment. SDS (50 μl, 5%) was added and the supernatants were incubated with 0.4 μg/ml RNase at 56 °C for 2 h to remove the cellular RNA. Proteinase K (1.5 μg/ml) was then added to the supernatant at 56 °C and it was further incubated for 2 h to remove the proteins. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volume of ice cold absolute ethanol. After centrifugation, the DNA pellet was washed with 70% ethanol and then air dried. The dried pellet was resuspended in 20 μl TE buffer (10 mM Tris–HCl, pH 7.5 and 0.1 mM EDTA) and incubated at 65 °C for 5 min. Finally the resuspended DNA was subjected to electrophoresis on a 2% agarose gel at a constant voltage of 40 V for 1–2 h.

3. Results

3.1. Doxorubicin induces phosphorylation of $p53Ser^{15}$ and apoptosis in human leukemic cells

The tumor suppressor protein, p53 accumulates in response to genotoxic effects such as chemotherapeutic agents [10], ionizing radiation [28], and UV light [29]. Once activated, p53 in turn mediates DNA repair, cell cycle arrest, and apoptosis [30–32]. It has been reported that the Ser^{15} phosphorylation site of p53 is exclusively linked to apoptosis-induction by chemotherapeutic drugs, and chemopreventive agents [32]. Therefore, we first examined the involvement of p53 phosphorylation at Ser^{15} up on Doxorubicin treatment on leukemic cells such as Jurkat and Molt-4 cells. Treatment of leukemic cells with different concentrations of Doxorubicin resulted in a dose dependent accumulation of Ser^{15} phosphorylated p53 in human leukemic cells (Fig. 1A). Next the time required for the $p53Ser^{15}$ phosphorylation upon Doxorubicin treatment of leukemic cells was examined. Exposure of Jurkat cells to 0.4 μM Doxorubicin, and exposure of Molt-4 cells to 0.04 μM Doxorubicin, increased the time dependent accumulation of Ser^{15} phosphorylated p53 (Fig. 1B). In order to examine whether the

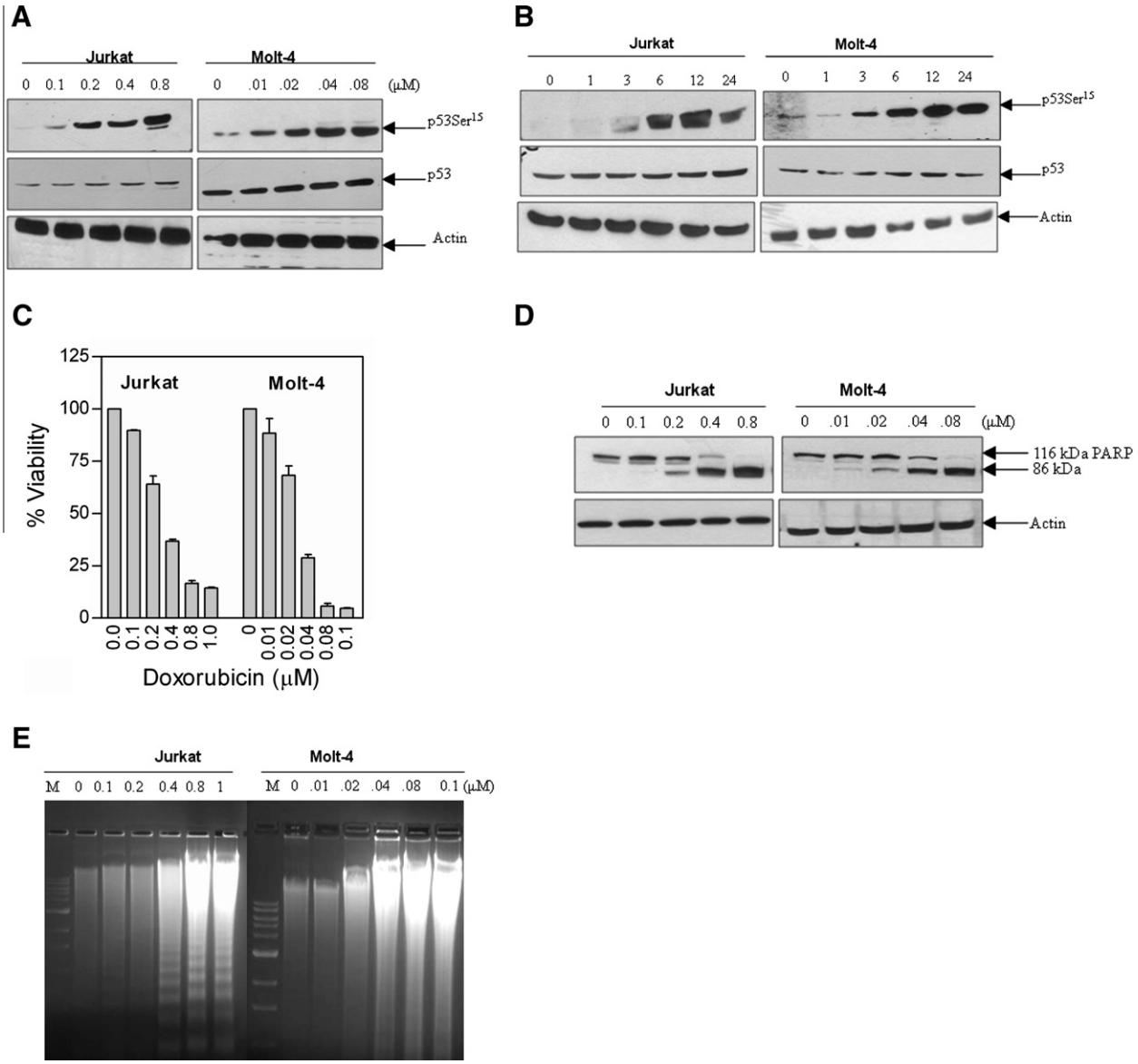

Fig. 1. Doxorubicin induces phosphorylation of p53 at Ser^{15} in human leukemic cells (A) Jurkat and Molt-4 cells were treated with the indicated concentrations of Doxorubicin for 24 h and (B) Doxorubicin (0.4 μM) for Jurkat, and (0.04 μM) for Molt-4 cells for the indicated time. Cells were lysed as described under Section 2, and were then fractionated by SDS–PAGE. Western blot were probed with antibodies specific for phospho-p53 (Ser15) and p53. Actin was used as a loading control. Blots shown here are representative of three independent experiments. (C) MTT cell viability assay for Jurkat and Molt-4 cells following treatment with indicated concentrations of Doxorubicin for 24 h. Data represent the mean ± SD ($n = 3$). (D) Jurkat and Molt-4 cells were treated with the indicated concentration of Doxorubicin and PARP cleavage was measured by Western blot analysis. Actin was used as a loading control. (E) Jurkat and Molt-4 cells were treated with the indicated concentrations of Doxorubicin for 24 h, and the DNA fragmentation analysis was performed as described under Section 2.

$p53Ser^{15}$ phosphorylation induced by Doxorubicin could lead to cell death, MTT assays were performed. Doxorubicin caused a dose dependent reduction in cell viability (Fig. 1C). The cytotoxic activity of Doxorubicin was, at least in part, attributable to apoptosis, as evidenced by the dose dependent PARP cleavage, and DNA fragmentation (Fig. 1D and E).

3.2. Doxorubicin induces caspase dependent Cer generation

Ceramide has been suggested to be a "tumor suppressor lipid" and its generation is induced exclusively by proapoptotic insult, and not during growth stimulation. It has been reported that Cer accumulates in response to chemotherapeutic agents, such as actinomycin D [10], butyric acid [33], as well as, Fas-induced apoptosis in leukemic cells [20]. Therefore, we examined the involvement of Cer generation upon Doxorubicin treatment on leukemic cells, including Jurkat and Molt-4 cells. Treatment of leukemic cells with Doxorubicin resulted in a dose and time dependent accumulation of ceramide (Fig. 2A and B).

Previous studies have suggested that caspases, a family of cysteine-dependent aspartate directed proteases, are important mediators of apoptosis [34]. To determine the role of caspases in Doxorubicin-induced Cer generation and apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk. When human leukemic cells were pre-treated with z-VAD-fmk, and then they were treated with Doxorubicin for 24 h, Cer generation (Fig. 2C), and apoptosis induction were significantly inhibited (Fig. 4D). This data indicates that Cer generation is tightly regulated and z-VAD-fmk-sensitive caspases are critical for the Doxorubicin-induced apoptosis in human leukemic cells.

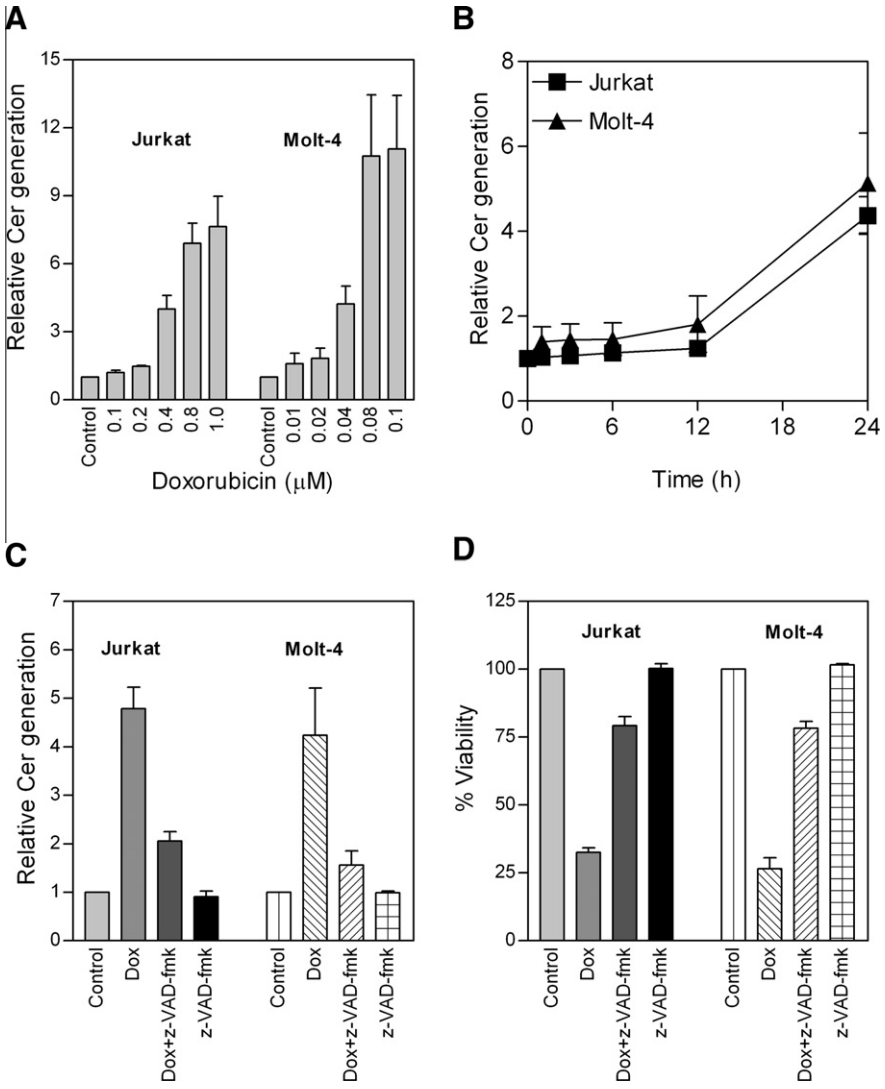

Fig. 2. Doxorubicin induces the caspase dependent Cer generation and apoptosis human leukemic cells (A) Jurkat and Molt-4 cells were treated with the indicated concentrations of Doxorubicin for 24 h and (B) Doxorubicin (0.4 μM) for Jurkat and (0.04 μM) for Molt-4 cells for the indicated time. Intracellular Cer were measured as described under Section 2. (C) Cells were pre-treated with 50 μM concentration z-VAD-fmk for 1 h, followed by incubation with Doxorubicin (0.4 μM) for Jurkat and (0.04 μM) for Molt-4 cells for additional 24 h, cellular lipids were extracted and assayed for Cer by HPLC method as described in Section 2. (D) Cells were pre-treated with 50 μM concentration z-VAD-fmk for 1 h, followed by incubation with Doxorubicin (0.4 μM) for Jurkat and (0.04 μM) for Molt-4 cells for additional 24 h, cell viability were measure by MTT assay.

3.3. Effects of GSH on Doxorubicin induced apoptosis

The antioxidant GSH plays an important role in scavenging reactive oxygen species and the detoxification process [3]. The exact molecular mechanism of the protective effect of this antioxidant against Doxorubicin-induced apoptosis is not known. Therefore, we investigated whether GSH can protect cells against Doxorubicin-induced cell death. Pre-treatment of leukemic cells with 10 mM GSH strongly suppressed the Doxorubicin induced PARP cleavage (Fig. 3A), DNA fragmentation (Fig. 3B), and cell death (Fig. 3C). These results indicate that GSH inhibits Doxorubicin-induced cell death in leukemic cells.

3.4. Effects of GSH on Doxorubicin induced p53Ser^{15} phosphorylation and Cer generation

In order to evaluate the protective mechanism(s) of GSH on Doxorubicin-induced apoptosis, the influence of GSH on Doxorubicin-induced p53Ser^{15} phosphorylation and Cer generation were examined. As shown in the Fig. 4A and B, pre-treatment of the cells with GSH inhibited Doxorubicin-induced p53Ser^{15} phosphorylation and Cer generation. These findings indicate that GSH may, indeed, mediate its anti-apoptotic effects via inhibition of p53Ser^{15} phosphorylation and Cer generation in Doxorubicin-induced apoptosis in human leukemic cells.

4. Discussion

Doxorubicin is one of the major anti-cancer drugs widely used in the treatment of acute myeloid leukemia. However, the signalling pathways triggered by this agent in leukemic cells are not completely understood. The present study demonstrates that GSH is an important regulator of Doxorubicin-induced human leukemic cell death. Specifically, we show that GSH inhibits p53Ser^{15} phosphorylation, caspase dependent Cer generation, PARP cleavage, and DNA fragmentation, all of which lead to a significant protection against Doxorubicin-induced apoptosis.

The tumor suppressor protein p53 is a transcription factor that enhances several genes known to play a critical role in transducing apoptotic signals [31] and mediates cell cycle arrest and apoptosis.

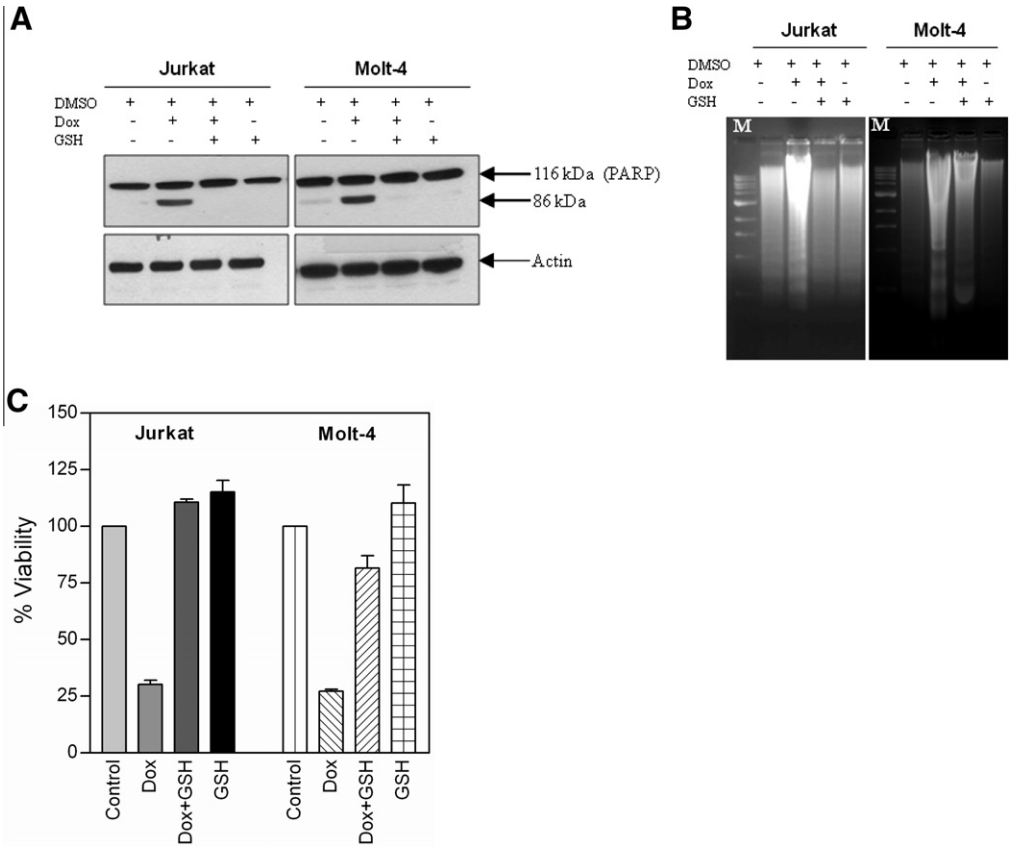

Fig. 3. Extracellular supplementation of GSH prevents Doxorubicin induced PARP cleavage, DNA fragmentation and loss of viability. Cells were pre-treated with 10 mM concentration GSH for 1 h, followed by incubation with Doxorubicin (0.4 μM) for Jurkat and (0.04 μM) for Molt-4 cells. (A) PARP cleavage was measured by Western blot analysis. Actin was used as a loading control. Blots shown here are representative of three independent experiments. (B) DNA fragmentation was measured as described under Section 2. (C) Cell viability was measured by MTT assay.

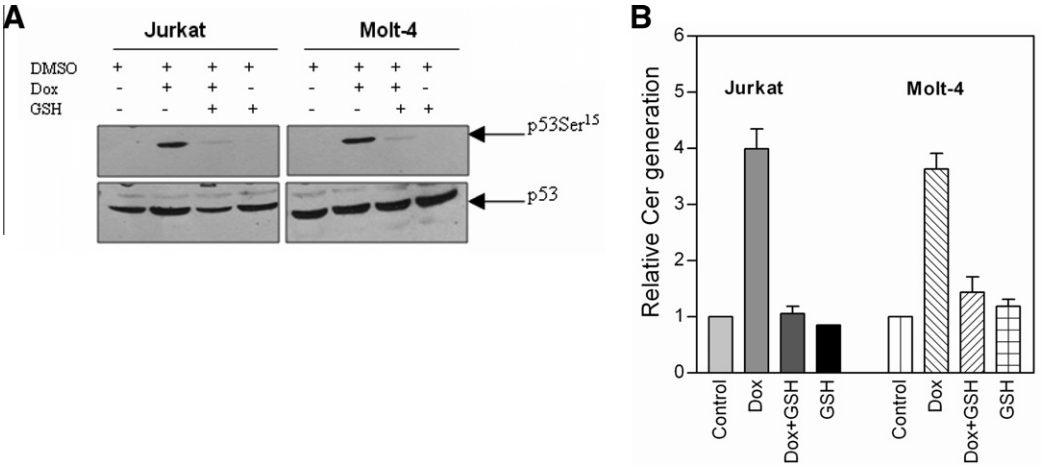

Fig. 4. Extracellular supplementation of GSH prevents Doxorubicin induced p53Ser^{15} phosphorylation and Cer generation. Cells were pre-treated with 10 mM concentration GSH for 1 h, followed by incubation with Doxorubicin (0.4 μM) for Jurkat and (0.04 μM) for Molt-4 cells. (A) p53Ser^{15} phosphorylation were measured by Western blot analysis. (B) Cellular lipids were extracted and assayed for Cer by HPLC method as described in Section 2.

Its expression and activity are elevated in response to ionising radiation, UV light, and curcumin [3,5,28]. In our study, we found that Doxorubicin has an immediate effect on p53 signalling pathway. As can be seen in Fig. 1A Doxorubicin induces the phosphorylation of p53 at Ser^{15}. These results are in line with a previous study on the effect of Doxorubicin on p53 in rat myoblastic H9c2 cells [35]. The redox environment of cells has been suggested to be an important regulator of apoptosis. Small thiols, including GSH are considered as protective antioxidants acting as free radical scavengers in response to oxidative damage; thus GSH maintains the redox balance in the cells [36–38]. Our data is in agreement with these findings, and demonstrate that Doxorubicin induced phosphorylation of p53Ser^{15} is inhibited with extracellular GSH supplementation.

In this study, Cer content increases in a time and dose dependent manner following Doxorubicin treatment of human leukemic cells (Fig. 2A and B). This proapoptotic sphingolipid is produced by cancer cells in response to exposure to ionizing radiation and most

chemotherapeutic drugs, however, some chemo- and radio-resistant tumor cells exhibit defect in Cer generation, supporting the emerging role of Cer as a tumor suppressor lipid [10,18,39,40]. Many cellular proteins such as caspases are essential for the execution of cell death by apoptotic stimuli [34]. For cells under growing apoptosis, caspases are shown to be either upstream or downstream of Cer accumulation [41,18,19]. Moreover, lipid enzymes potentially targeted by caspases have recently been identified [19,20]. In our study, we demonstrate that inhibition of multiple caspases using z-VAD-fmk inhibits Doxorubicin-induced Cer production, PARP cleavage and apoptosis. These data suggest that caspase dependant increase of Cer is the prime signaling event in the Doxorubicin-induced apoptosis in human leukemic cells. Furthermore, our data also demonstrate that cell pretreatment with exogenous GSH, prevented the Doxorubicin-induced Cer generation, PAPR cleavage, DNA fragmentation, and apoptosis. Our data strongly suggest a pivotal role for GSH in Doxorubicin-induced Cer-generation and apoptosis signaling in human leukemic cells.

In conclusion, the results obtained in the present study leads us to propose that in human leukemic cells, apoptosis induced by Doxorubicin is associated with p53Ser^{15} phosphorylation, and caspase dependent Cer production. These effects can be counteracted by extracellular supplementation of GSH, resulting in the inhibition of p53Ser^{15} phosphorylation and caspase dependent Cer accumulation, subsequently, resulting in inhibition of Doxorubicin-induced apoptosis. Altogether, these results emphasize that the cellular antioxidant defense mechanism can influence the clinical efficacy of Doxorubicin by modulating tumor suppressor protein and tumor suppressor lipid-mediated apoptotic signaling in human leukemic cells. These findings are important for a better understanding of the mechanism of action of chemotherapeutic agents.

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