ENDOGENOUS THIOREDOXIN IS REQUIRED FOR REDOX-CYCLING OF ANTHRACYCLINES AND P53-DEPENDENT APOPTOSIS IN CANCER CELLS

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Running Title: Thioredoxin enhances apoptosis

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Apoptosis is a major mechanism of cancer cell destruction by chemotherapy and radiotherapy. Anthracycline class of antitumor drugs undergo redox-cycling in living cells producing increased amounts of reactive oxygen species and semiquinone radical, both of which can cause DNA damage, and consequently trigger apoptotic death of cancer cells. We show here that MCF-7 cells overexpressing thioredoxin (Trx) were more apoptotic in response to daunomycin. Trx overexpression in MCF-7 cells increased the generation of superoxide anion (O_2^-) in anthracycline-treated cell extracts. Enhanced generation of O_2^- in response to daunomycin in Trx-overexpressing MCF-7 cells was inhibited by DPIC, a general NADPH reductase inhibitor, demonstrating that Trx provides reducing equivalents to a bioreductive enzyme for redox-cycling of daunomycin. Additionally, Trx increased p53 DNA binding and expression in response to anthracyclines. MCF-7 cells expressing mutant redox-inactive Trx showed decreased superoxide generation, apoptosis and p53 protein and DNA binding. In addition, down regulation of endogenous Trx expression by siRNA resulted in decreased expression of caspase-7 and cleaved PARP expression in response to daunomycin. These results suggest that endogenous Trx is required for anthracycline-mediated apoptosis of breast cancer cells. Taken together, our data demonstrate a novel pro-oxidant and pro-apoptotic role of Trx in anthracycline-mediated apoptosis in anthracycline chemotherapy.

Thioredoxin (Trx) is a low molecular weight protein (12 kD) that is widely distributed; Trx is found within the cytoplasmic, membrane, extra cellular and mitochondrial cellular fractions (1,2). The Trx system includes Trx, and Trx reductase (TR) and peroxiredoxins. TR is an efficient protein disulfide reductase that uses NADPH as a source of reducing equivalents. Besides being an antioxidant itself (3,4), Trx also plays an important role in regulating the expression of other antioxidant gene such as manganese superoxide dismutase (5). Trx overexpression also enhances the expression of peroxiredoxin that could reduce peroxides to molecular oxygen and H_2O (6). Trx has been shown to regenerate oxidatively inactivated proteins (7,8). In addition to its role as an antioxidant protein, Trx has been shown to have growth promoting properties (9). In contrast, a recent study has demonstrated that overexpression of redox-active Trx could promote cell death via activation of caspase-8 (10). Additional studies have shown that TR is critical for cell death, and a Trx-dependent mechanism has been suggested (11). Recent studies also indicate that caspases, the executioner of cell death by apoptosis could be activated by Trx due to its disulfide reducing properties (12). Caspases are rich in cysteine motif that is required for its catalytic activity. Therefore, oxidation could inhibit caspase activity, which could be restored by Trx system. (13). Furthermore, Trx has also been shown to promote p53 DNA binding due to its reducing actions on DNA binding cysteine motifs on p53 (14). Taken together, accumulating evidence suggest that Trx is a multi-functional protein, which can participate in proliferation, as well as cell death process. The antioxidative action of Trx could be due to its MnSOD inducing properties (5,15), as well as direct scavenging of hydroxyl radicals or singlet oxygen.

Anthracycline class of anticancer drugs such as doxorubicin or daunomycin has been shown to induce p53-dependent apoptosis in cancer cells
Additionally, anthracyclines have also been shown to cause DNA damage, which increases p53 expression (18,19). p53 is a sequence-specific transcription factor, which can induce pro-apoptotic or suppress anti-apoptotic genes in response to DNA damage or irreparable cell cycle arrest (20). Phosphorylation of p53 on ser-15 residue dissociates MDM2 and activates p53 as a transcription factor, which binds to various p53-dependent genes resulting their activation or repression (20). While evaluating the protective effect of Trx in daunomycin-induced cytotoxicity we observed increased death of MCF-7 cells overexpressing Trx. Since Trx has been shown to protect against oxidative stress and daunomycin-mediated cytotoxicity has been shown to be mediated in part by ROS, our observation was rather surprising and novel. Anthracyclines contain quinone moieties in their structure, which can undergo biochemical reduction by one or two electrons catalyzed by flavoenzymes in the cell using NADPH as an electron donor (21-23). This bio-reductive process generates semiquinone radical with concomitant production of superoxide anion (O$_2^-$). The semiquinone radical intercalates with the DNA resulting in DNA damage. The formation of O$_2^-$ is the beginning of a cascade that generates hydrogen peroxide and hydroxyl radicals, generally referred to as reactive oxygen species (24). In addition to various bioreductive enzymes, low molecular weight protein or non-protein thiols may also take part in the redox cycling process (25).

In the present investigation we report that endogenous Trx is required for daunomycin-induced apoptosis of cancer cells. In addition, we also demonstrate that Trx enhances the apoptotic death of cancer cells in response to daunomycin due to enhanced redox-cycling of anthracyclines. In contrast, cells that express redox-inactive Trx or transfected with Trx siRNA show resistance to apoptosis.

**Experimental Procedure**

**Reagents**

Daunomycin was purchased from Sigma (St.Louis, MO) and 5-iminodaunomycin was obtained from National Cancer Institute (NCI). Anti-p53 (full length), anti-caspase 7, and anti-caspase-1 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA); anti-p53 phosphoser-15, anti-caspase-6, anti-caspase-8 (recognizes cleaved fragment) and anti-Poly ADP Ribose Polymerase (PARP) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-thioredoxin antibody was purchased from Research Diagnostics (Flanders, NJ).

**Cell culture and adenovirus production**

MCF7 cells were cultured in DMEM with 10% fetal bovine serum and 100 units of penicillin/streptomycin. MCF7 clones expressing Trx (Trx9), dominant negative redox inactive Trx (Sreb4) and only vector (Vector) were generous contribution of Dr. Garth Powis (Arizona Cancer Center, Tucson, Arizona) and have been described (26,27). MCF-7 clones were cultured in DMEM containing G418 (300 µg/ml). A549 cells were obtained from ATCC and propagated in F12K medium. AdenoX system was obtained from Stratagene Corporation (La Jolla, California) and Trx or mutant Trx ORF (26) was cloned into pAdenoX vector. Recombinant virus was allowed to infect HEK293 cells for generation of viral particles. For transfection, MCF-7 cells were infected with approximately 1X10$^8$ infectious units (per million cells) and after 48 hours protein expression was determined using ELISA.

**RNA interference:**

p53, Trx and scrambled non-targeting siRNA were purchased from Dharmacon RNA technologies (Lafayette, CO). For transfection, MCF-7 or A549 cells were seeded in 35 mm dishes to obtain 20% confluency at the time of transfection. Xtreme siRNA transfection reagent (Roche Molecular Diagnostics, Indianapolis, IN) was used to transfect siRNA to a final concentration of 100 nM. Inhibition of gene expression by siRNA was determined after 72 hours by western analysis.

**Thioredoxin Activity Assay:**

Thioredoxin activity assay was performed as described by Holmgren et al. (1). Briefly, the reaction mixture contained NADPH (200 µM), porcine insulin (80 µM; Sigma), and bovine TR (0.1 µM) in 0.05 M potassium phosphate buffer (pH 7.0) containing EDTA (1 mM) in a total volume of 0.5 ml. The reaction was started by addition of bovine TR (0.1 µM). TRX activity
was calculated as μmoles of NADPH oxidized per minute per mg protein at 25°C (Beckman DU800 spectrophotometer).

**TUNEL assay:**
Apoptotic cells were detected using In Situ Cell Death detection, POD kit (Roche Molecular Biochemicals, Indianapolis, Indiana). Apoptotic DNA strand breaks were identified by labeling 3’-OH termini with fluorescein-dUTP using Terminal deoxy Transferase as per manufacturer’s protocol. Cells were allowed to adhere overnight in chambered glass slides (Nunc) to a final density of 25,000 cells per well. Following treatment with appropriate concentration of drugs media was removed, and cells were washed twice with PBS containing 1% BSA and fixed in 4% paraformaldehyde for 30 min. Cells were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 mins on ice, and washed twice with PBS containing 1% BSA. The labeling reaction was performed using FITC labeled dUTP along with other nucleotides by terminal deoxynucleotidyl transferase for 60 mins in dark at 37°C in humidified chamber. Further, cells were washed with PBS-1% BSA, mounted and the incorporated fluorescein-dUTP was analyzed using fluorescent microscope (Carl Zeiss, Axiovert M200).

**Flow cytometry:**
Cells were treated with drugs for 48 hours. Floating cells were collected, and adherent cells were washed with PBS and trypsinized. Floating and adherent cells were pooled and centrifuged at 500 X g for 3 mins. Cells were washed again with PBS containing 1% FBS and resuspended in 500 ml of PBS followed by fixing in 7.5 ml of ice-cold ethanol (70%), added drop wise while vortexing and stored in -20°C overnight. After two washes with PBS containing 1% FBS, cells were resuspended in the same buffer and stained with Propidium Iodide 10 mg/ml (Sigma, St.Louis, USA) in the presence of RNase 250 mg/ml at 37°C for 30 minutes in dark. Stained cells were analyzed using Epics Elite ESP Coulter, using argon laser at 488 nm wavelength. Flow cytometric results were analyzed and apoptosis was defined as ‘sub G1’ peak (6) using Multicycle software.

**Western blotting:**
Protein lysates were prepared using radio immunoprecipitation assay (RIPA) buffer containing 5% sodium deoxycholate, 1% SDS, 1% Igepal in PBS with protease inhibitors and protein concentration was determined using Biorad protein assay reagent (Biorad). Equal amounts of protein was resolved on 10% SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). The blot was treated with appropriate dilutions of primary antibody and visualized using either Lumiglo (Cell Signaling Technology, Beverly, MA) or ECL plus system (Amersham Pharmacia Biotech, Piscataway, New Jersey) with appropriate HRP conjugated secondary antibody.

**Determination of O2\(^{-}\) production by reduction of ferricytochrome c:**
Superoxide production was measured as superoxide dismutase (SOD) inhibitable reduction of ferricytochrome c (28). Cells were sonicated in potassium phosphate buffer (0.05M, pH 7.8 plus 1 mM EDTA), centrifuged and the supernatant was used for the assay. To determine the O2\(^{-}\) generation in the cell lysate, the supernatant was incubated with 10 μM drug and 10 μM cytochrome c, with or without 1unit of SOD to determine SOD inhibitable rate. All reactions were performed in triplicate. The reduction of ferricytochrome c was measured both in kinetic and end point mode with path check on for 1hour duration at a wavelength 550 nM using Spectramax 190 plate reader (Molecular Devices). Total protein was quantified using Bradford protein assay (Biorad).

**In situ detection of O2\(^{-}\) by fluorescent probe DiCarboxyFluorescein-DiAcetate (DCF-DA).**
Cells were grown in chambered glass slides (Nunc) to a final density of 25,000 cells per well. Cells were pre-incubated with 20 μM DCF-DA (Sigma, St. Louis, Missouri) in 20 mM HEPES in PBS containing BSA, (5mg/ml) at 37°C for 30 minutes followed by washing with PBS buffer, and the drug was added and observed for 300 seconds in a Nikon laser confocal microscope using laser beam wavelength 488 nm analyzed by Ultraview software (Perkin-Elmer).
**Clonogenic assay**

Cells were trypsinized and seeded to a final density of 1 X 10^6 viable cells per 100 mm dishes and allowed to attach overnight. Cells were then treated with appropriate concentration of drugs for 24 hours, trypsinized and seeded to a final density of 500,000 viable cells per 100 mm dish. Viable cells were determined using Vicell counter (Beckman Coulter). After 14 days, the surviving colonies were washed in PBS, fixed in 70% ethanol and stained using 0.1% crystal violet in 90% ethanol. Colony containing a minimum of 30 cells were counted using colony counting feature present in the quantity one software from Biorad. The assays were performed in triplicate and the data was statistically analyzed using instat 2.01 software.

**Nuclear Extract preparation**

Nuclear extract was prepared as described previously (29). Briefly, cells were washed in ice cold PBS and harvested in 2 ml of ice cold PBS by centrifugation. Cell pellets were resuspended in 400 µl of Buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1mM EDTA, 1 mM Dithiothreitol, 1mM Phenylmethysulfonyl fluoride (PMSF) and 50 µg/ml of leupeptin and antipain by gentle pipetting. Cells were allowed to swell on ice for 15 mins followed by addition of 25 ml 10% Nonidet-P40 and vortexed at full speed for 10 seconds. The homogenate was centrifuged for 30s at 14,000 rpm. The nuclear pellet was resuspended in buffer C (20 mM HEPES, pH 7.8, 0.42M NaCl, 5 mM EDTA, 1mM Dithiothreitol, 1mM PMSF in 10% v/v glycerol) and tubes were rocked gently at 4°C for 30 mins on a shaking platform. The extracts were then centrifuged at 14,000 rpm for 25 mins, and the supernatant was saved as nuclear extract in -70°C for further experiments. Protein was quantified using Bradford protein assay (Biorad).

**Electrophoretic Mobility shift assay (EMSA)**

For the EMSA, the p53 consensus oligonucleotide was obtained from Genosys (5’-GGCATGTCCGGCATGTC-3’), and was end labeled using T4 Poly nucleotide kinase (New England Biolabs, Beverly, MA) and [γ-32P] ATP (Perkin Elmer, Boston, MA) in 10X kinase buffer supplied with the enzyme. Ten microgram of nuclear protein was pre-incubated in 5µl of 5X binding buffer (20% glycerol, 5 mM MgCl2, 5 mM EDTA, 5 mM DTT, 500 mM NaCl, 50 mM Tris HCl, 0.4 mg/ml calf thymus DNA), 200 ng anti-p53 pAb 421 and 2 µg of poly dIdC for 15 mins followed by binding with labeled oligonucleotide for 30 mins. The nuclear protein was separated by electrophoresis using 4% native polyacrylamide gel and 0.25X of TBE (Tris-Borate- EDTA) as running buffer. Gels were dried and exposed to Kodax Biomax X-ray film overnight.

**Thioredoxin ELISA**

Cells were homogenized in 50 mM Tris'HCl (pH 7.5) containing EDTA (1mM), PMSF (1 mM), leupeptin (20 µg/ml), and antipain (20 µg/ml). Lysates were microcentrifuged for 10 min at 14000 rpm. The protein concentration in the supernatant was measured using the Bradford method (Biorad) with bovine serum albumin as standard. ELISA was performed as previously described (30).

**Statistical Analysis.**

All statistical analysis was performed using In Stat software program (V2.01, and 3.0). All experiments were repeated at least twice.

**Results**

**Increased expression of redox-active Trx enhances apoptosis in response to daunomycin.**

To test whether Trx overexpression protects MCF-7 cells against daunomycin mediated apoptosis, we treated vector, Trx9 or serb4 cells (clones of MCF-7 cells) with daunomycin and determined apoptosis as described in ‘Experimental Procedures’. First we determined apoptosis using TUNEL assay, which detects nicks in the DNA that are generated during DNA damage and apoptosis. We expected to find more TUNEL-positive nuclei in Serb4 cells compared to Trx9 cells in response to daunomycin, since previous studies show that Trx could protect against the cytotoxic actions of other anticancer drugs such as cisplatin and bleomycin (31-33). However, to our surprise we observed a higher number of TUNEL positive Trx9 cells (Fig 1A & B), in response to daunomycin. TUNEL assay detects nicks generated by drug-induced DNA damage and endonuclease activation during apoptosis. MCF-7...
cells lack caspase 3 and do not undergo classical DNA laddering during apoptosis. Therefore, we further determined apoptotic cells as ‘Sub-G1’ population by flow cytometry by propidium iodide staining. Treatment of vector, Trx9 or serb4 clones with daunomycin resulted in the appearance of apoptotic cells (Fig. 1 C & D). Trx9 cells showed a higher percentage of apoptotic cells (23%) compared to vector or Serb4 cells (8%). These data also agree with our TUNEL data that show increased apoptosis of Trx9 cells in response to daunomycin. Trx activity was assayed in vector, serb4 and Trx9 cells using insulin reduction assay as described in experimental procedure (Fig 1E).

Decrease clonogenic survival of MCF-7 cells overexpressing Trx.

After cytotoxic treatment, cells can survive DNA damage through various repair processes and may continue to propagate into colonies (34,35). Therefore we compared the clonogenic survival of Trx9, serb4 or vector cells treated with daunomycin at concentrations as low as 0.025 µM. A clonogenic assay is a more stringent assessment of chemosensitivity than TUNEL or Sub-G1 peak measurements (34,35). Trx9 cells treated with daunomycin formed significantly less colonies at the end of 14 days, whereas vector cells treated with daunomycin formed several colonies (Fig. 2A). Thus MCF-7 cells overexpressing Trx exhibited increased apoptosis and decreased clonogenic survival in the presence of daunomycin, as compared with vector-transfected cells.

In addition to MCF-7 clones, we also generated Trx or dnTrx expressing clones in A549 cells to test whether the observations with MCF-7 cells could be reproduced in other cell types. As demonstrated in Fig 2B, A549-vector only clones or A549-dnTrx clones showed several colonies at the end of 14 days, whereas A549-Trx clones did not show significant number of colonies. These data demonstrate that either A549 cells or MCF-7 cells expressing higher level of Trx undergo increased apoptosis and decreased clonogenic survival in response to daunomycin. Next, to determine whether endogenous Trx contributes to apoptosis induced by anthracyclines, we used RNA interference to down regulate Trx expression.

Silencing Trx expression by siRNA decreases apoptosis in MCF-7 cells, as well as in A549 cells in response to daunomycin.

The apoptosis experiments described above were done using Trx or dnTrx overexpressing clones of MCF-7 cells. Therefore, there is reason to believe that the data obtained could be specifically applicable to a specific clone of Trx or dnTrx of MCF-7 cells. In addition, it is not clear whether effects that were observed is only related to the overexpression of Trx. Therefore, to understand the role of endogenous Trx in apoptosis induced by daunomycin, we down regulated the level of Trx in MCF-7 cells using siRNA approach. As shown in Fig 3A, treatment of MCF-7 cells with Trx siRNA down regulated Trx protein level. The decrease was about 95% compared to cells transfected with non-targeting siRNA. Following siRNA transfection, cells were treated with daunomycin and the level of apoptotic markers were evaluated by western analysis. As shown in Fig 3B (top panel) and Fig 3C, MCF-7 cells transfected with non-targeting siRNA and treated with daunomycin demonstrated a significant increase in p53 protein level. In contrast, cells treated with Trx siRNA demonstrated a significantly (p<0.001) lower level of p53 protein in response to daunomycin treatment (Fig 3C). Additionally, the level of active caspase-7 (Fig 3B, 2nd panel and Fig 3D), and the level of cleaved PARP (Fig 3B 3rd panel; Fig 3E) were also significantly (p<0.001) decreased in Trx siRNA transfected cells compared to non-targeting siRNA transfected cells. These data demonstrate a crucial role of endogenous Trx in daunomycin-induced apoptosis in MCF-7 cells.

Recent studies have demonstrated that caspases-1 expression is up regulated in response to doxorubicin in a p53-dependent manner in MCF-7 cells (36,37). Since caspases-1 expression is pro-apoptotic, and its expression is dependent on p53, we examined the level of caspases-1 in response to daunomycin treatment and the effect of silencing Trx on the expression of caspases-1. In addition, we also examined the expression of caspases-6 and cleaved caspases-8 expression in response to Trx silencing in daunomycin treated MCF-7 cells. As demonstrated in Fig 3F and Fig 3G, caspases-1 expression was significantly up regulated in
response to daunomycin in MCF-7 cells transfected with non-targeting siRNA. In contrast, cells transfected with Trx siRNA demonstrated significant inhibition of caspases-1 up regulation in response to daunomycin. However, the level of caspases-6 remained unchanged in response to daunomycin (Fig 3F, middle panel). Treatment of cells with daunomycin resulted in the appearance of cleaved caspases-8 product (Fig 3F, lower panel), but the level of cleaved caspases-8 level remain unchanged in Trx siRNA treated cells compared to non-targeting siRNA treated cells. Taken together, these data suggest that caspases-1 and caspases-7 levels are modulated by daunomycin treatment, and Trx plays an important role in regulation of expression of these caspases.

In order to determine whether the role played by Trx in MCF-7 cells could be reproduced in other cell lines, we transfected lung adenocarcinoma cells A549 with siRNA for Trx or a non-targeting siRNA sequence, and treated these cells with daunomycin. As demonstrated in Fig 4A, transfection of Trx siRNA significantly down regulated Trx level in A549 cells. These cells were treated with daunomycin and the level of p53 was determined by quantitative immunoblotting. As shown in Fig 4B and C, down regulation of Trx protein levels by siRNA significantly lowered p53 protein level similar to that observed with MCF-7 cells. Taken together, these data demonstrate that endogenous Trx is required for daunomycin-induced apoptosis of cancer cells. Next, we determined how p53 expression is modulated in the presence of higher level of redox-active Trx or in the absence of redox-active Trx.

**MCF-7 cells expressing higher Trx activity show higher p53 protein levels in response to daunomycin**

Anticancer agents that induce DNA damage also induce p53-mediated apoptosis (38). In the event of DNA damage, p53 is activated by phosphorylation and binds to the consensus sequence of the DNA, resulting in the regulation of gene expression required for apoptosis or cell cycle arrest (39). Phosphorylation of p53 blocks Mdm-2 binding and thereby prevents degradation of p53 protein resulting in accumulation of p53 (40). To determine the role of Trx in daunomycin-induced p53 protein expression, we examined the level and the extent of phosphorylation of p53 in Trx9 cells. Treatment of cells with 1 µM daunomycin increased p53 protein in all clones (Fig. 5A, upper panel); however, in Trx 9 cells there was 2-3 fold increase in p53 protein level than in vector or Serb4 cells. Phosphorylation of p53 at ser15 has been shown to be crucial in activating p53 as a transcription factor (17). Conformational changes due to phosphorylation is required for its DNA binding activity (41). Therefore, we next, evaluated the phosphorylation state of p53 on ser15 in daunomycin-treated cells using phospho-specific antibodies (Ser-15). Trx9 cells exhibited higher phospho-p53 (Ser-15) level in response to daunomycin compared to vector or Serb4 cells, suggesting that higher level of Trx could be potentiating the apoptotic potential of daunomycin (Fig. 5A, lower panel).

To determine whether the results obtained with a specific clone overexpressing Trx could be reproduced in other clones of MCF-7 cells overexpressing Trx, we generated several clones of Trx and dnTrx as described in the experimental procedure section in MCF-7 cells as shown in Fig 5B. These clones were treated with 1 µM daunomycin for 24 h followed by detection of p53 expression using western analysis. As shown in Fig 5C MCF-7 clones Trx25 and Trx26 showed higher p53 expression in response to daunomycin. In contrast, dnTrx clones 1 & 4 did not show higher p53 expression compared to vector only clones or Trx clones. These studies show that the results observed in vector, Trx9 or Serb4 cells could be reproduced in other MCF-7 clones. Therefore, using different clones of MCF-7 cells or other cells (A549) we observed increased p53 expression in response to daunomycin in Trx overexpressed cells.

Next, we examined whether transient overexpression of Trx in MCF-7 cells could produce similar results obtained using stable clones, because there is reason to believe that stable expression of a protein could be clone-specific. Additionally, the physiological response of stable clones could be different than that of transient overexpression. We used adenovirus mediated gene delivery, as well as used lipofectamine-mediated transfection to overexpress Trx or dnTrx in MCF-7 cells and
studied its effect on p53 expression in response to daunomycin. As demonstrated in Fig 5D, adenovirus infection increased Trx or dnTrx level in MCF-7 cells. When these cells were treated with daunomycin increased p53 expression was observed in MCF-7 cells overexpressing redox-active Trx (Fig 5E). In contrast, dnTrx-overexpressing cells demonstrated almost no induction of p53 (Fig 5E). Additionally, as shown in Fig 5F, transient transfection also increased Trx or dnTrx levels in MCF-7 cells (data not shown). When these cells were treated with daunomycin we observed similar pattern of p53 expression (Fig 5F). Taken together, these data indicate that overexpression of Trx enhances p53 expression in response to daunomycin, whereas expression redox-inactive Trx inhibits the expression of p53 in response to daunomycin.

**p53 DNA binding is enhanced in Trx9 cells in response to daunomycin:**

Since phosphorylation of p53 activates its DNA binding that results in the regulation of the expression of many different genes involved in apoptosis or DNA repair, we used electrophoretic mobility shift assays (EMSA) to measure p53 binding to the DNA in nuclear extracts of cells treated with or without daunomycin. As demonstrated in Fig 6A, daunomycin-induced p53 DNA binding was several folds higher in Trx9 cells than in vector or serb4 cells. Increased DNA binding implies increased transcription of p53-dependent genes, which may lead to increased apoptosis. We also determined the p53 DNA binding in MCF-7 cells transiently overexpressing Trx or its mutant form. As shown in Fig. 6B, higher levels of p53 DNA binding was observed in cells expressing higher levels of Trx. These studies indicate that higher Trx level could enhance the transcription of p53-dependent genes that could enhance apoptosis.

**Silencing p53 decreases expression of apoptotic proteins such as active caspase-7 or cleaved PARP.**

Since daunomycin treatment in Trx-siRNA transfected cells resulted in decreased p53 accumulation and apoptosis, we sought to determine whether the observed decrease in apoptosis in daunomycin is dependent on the level of p53 accumulation. We used p53 siRNA to inhibit p53 expression in MCF-7 cells (Fig 7, upper panel), and treated these cells with daunomycin. Transfection of MCF-7 cells with p53 siRNA resulted in about 90% decrease in the expression of p53 (Fig 7A, upper panel and Fig 7B). We also observed a decrease in the level of caspase 7 (Fig 7A middle panel and Fig 7C) and a decrease PARP cleavage (82 kDa cleaved PARP; Fig 6A, third panel, and Fig 7D) in p53 siRNA transfected cells compared to non-targeting siRNA transfected cells. However cleavage of PARP was not completely inhibited in the presence of p53 siRNA suggesting that apoptotic degradation of PARP is partially dependant on p53. These data indicate that p53 is required for daunomycin-induced apoptosis in MCF-7 cells. Therefore, the data generated using Trx overexpression suggest that apoptotic potential of MCF-7 could be increased in the presence of higher levels of redox-active Trx.

Since daunomycin is a quinone containing anthracycline, and undergoes redox-cycling that generates reactive oxygen species (ROS) and the semiquinone radical, we tested whether Trx could contribute to enhanced redox cycling in vivo that could increase the expression of p53-mediated apoptosis.

**Daunomycin treatment increases the generation of ROS in Trx9 cells:**

We estimated the total load of oxidative stress by using the ROS-sensitive probe 2',7'-dichlorofluorescin diacetate (DCF-DA) to measure the total cellular peroxide levels in vector, Trx9 or Serb4 cells in response to daunomycin. Pre-incubation of cells with non-fluorescent DCF-DA dye followed by treatment with daunomycin resulted in enhanced fluorescence due to increased oxidation of DCF-DA in Trx9 cells compared to vector or serb4 cells (Fig 8 A & B). We also used 5-iminodaunomycin, a non-redox cycling form of daunomycin as a negative control (Fig 8A&C). 5-iminodaunomycin does not contain the quinone moiety of anthracyclines, and therefore does not generate superoxide anions (42). Treatment with 5-iminodaunomycin did not induce any detectable oxidation of DCF-DA in any of these Trx clones (Fig. 8 A & C). These data demonstrate that
daunomycin could undergo a higher rate of redox cycling in the presence of higher level of Trx.

Redox cycling of daunomycin generates semiquinone radical and $O_2^-$, both of which could induce DNA damage and cell death. Since Trx9 cells showed enhanced DNA damage and apoptosis, we sought to determine the rate of redox cycling of daunomycin in Trx9 cells. We measured the production of $O_2^-$ in the presence of daunomycin in Trx clones as an indicator of redox cycling (43). As shown in Fig. 8D, treatment of cells with daunomycin produced significantly higher level of $O_2^-$ in Trx9 cells compared to vector or Serb4 cells. We have previously shown that Trx being an antioxidant can scavenge hydroxyl radicals; however, it does not scavenge $O_2^-$ (4). In addition, we have also shown that *E. coli* Trx could participate in the redox cycling of anthracyclines generating $O_2^-$ (44). Therefore, we further investigated whether human Trx could also redox cycling daunomycin using additional controls. As demonstrated in Fig 8D, treatment of MCF-7 clones with 5-iminodaunomycin did not increase the generation of superoxide anions in Trx9 or Serb4 cells. These data demonstrate that quinone redox cycling is enhanced in the presence of increased level of Trx. Next, we evaluated the effect of transient overexpression of Trx on daunomycin redox cycling. As demonstrated in Fig 8E, MCF-7 cells expressing higher level of Trx in adenovirus-mediated overexpression generated higher level of superoxide anions compared to vector only or dnTrx transfected cells. In additional experiments (Fig 8F) we down regulated Trx expression using siRNA approach, and evaluated the response of these cells to daunomycin-mediated superoxide anion generation. As demonstrated in Fig 8G, there was significant inhibition of superoxide anion generation in cell with reduced level of Trx. These data indicate that endogenous Trx is required in cancer cells for redox-cycling of anthracyclines.

**Effect of bioreductive enzyme inhibitor on $O_2^-$ anion generation in Trx9 cells:**

We observed that Trx9 cells generate more $O_2^-$ in the presence of anthracyclines. Redox-cycling of anthracyclines has been shown to be mediated by one electron reduction by NADPH-cytochrome P-450 reductase (45), mitochondrial NADH dehydrogenase (46) and nitrate reductase (47) from *Neurospora*. Therefore, to determine the involvement of bioreductive enzyme(s) in the redox-cycling of daunomycin in the presence of increased thioredoxin we treated cells with diphenyleneiodonium chloride (DPIC) a non-specific inhibitor of NADPH-dependent reductases (48) As demonstrated in Fig 9, treatment of cells with DPIC inhibited generation of $O_2^-$ suggesting that Trx-mediated $O_2^-$ generation is dependant on bioreductive enzymes for anthracyclines redox-cycling. These results indicate that reductases could be involved in the redox-cycling of anthracyclines using reducing equivalents from reduced Trx.

**Redox cycling contributes to increased p53 DNA binding in the presence of higher level of redox-active Trx.**

Next, we determined whether the increase in p53-DNA binding observed in Trx9 cells depends on the redox cycling of daunomycin. By treating Trx clones with 5-iminodaunomycin, which cannot undergo redox cycling but intercalates with DNA we show that each of these clones vector, Trx9 and Serb4 exhibited enhanced p53-DNA binding (Fig 10A). However, there were minor differences in the intensity of DNA binding in these cells. To determine whether ROS mediate p53 DNA binding due to daunomycin redox-cycling we used $H_2O_2$ as a positive control for ROS, and Taxol as a negative control for ROS. Additionally, we used menadione as a positive control for quinone containing compound. As shown in Fig 10B, $H_2O_2$, Menadione and taxol treatment did not
In the present study we have demonstrated that increased expression of Trx enhances apoptosis in MCF-7 cells in response to daunomycin. In contrast, silencing endogenous Trx expression resulted in significant decrease in p53 expression and apoptosis in MCF-7 cells, as well as other cells in response to daunomycin. The expression and DNA binding of p53 protein was also increased in response to daunomycin in Trx9 cells compared to vector or Serb4 cells. Additionally, there was increased production of $O_2^-$ in extracts of Trx9 cells in response to daunomycin. DPIC, a general reductase inhibitor decreased the generation of $O_2^-$ as well as p53-DNA binding in Trx9 cells in response to daunomycin. Taken together, our studies demonstrate that Trx increases the redox-cycling of daunomycin and enhances the apoptotic potential of daunomycin. Our data also indicate that endogenous Trx is essential for anthracycline-dependant p53 expression and the activation of apoptotic cascade. These are novel pro-oxidant and pro-apoptotic role of Trx in response to anthracycline drugs. Additionally, the present study also indicate a crucial role of Trx in daunomycin-induced caspases-1 expression.

Thioredoxin is widely considered to be an antioxidant protecting cells from a variety of oxidative stress conditions (1,2). Our data indicate that Trx could provide reducing equivalents to bioreductive enzymes that can redox-cycle anthracyclines, and this process increases the apoptotic potential of anthracyclines. Interestingly, Trx has been shown to confer resistance against ROS-generating anticancer drugs due to its antioxidant properties (31,33). Conversely, recent studies have also shown that Trx does not confer resistance against doxorubicin (52). Furthermore, thioredoxin failed to protect MCF-7 cells from apoptosis induced by ROS generating drugs. The study published by Wang et al (53) is a correlative study that demonstrated a correlation between increased Trx expression in various leukemia cell lines and the drug resistance to adriamycin. Therefore, there was no mechanistic evaluation of the role of Trx in drug resistance or apoptosis. The study also compared several cell lines in terms of Trx expression and drug resistance. Our present investigation has shown, using different expression systems and
enhancing endogenous Trx in multiple cells that increased Trx expression could enhance the apoptotic potential of anthracyclines. Additionally, the role of endogenous Trx in apoptosis of cancer cells in response to daunomycin was clearly elucidated using siRNA approach. In contrast to the study of Wang et al, studies by Berggren et al (6) has clearly demonstrated that although Trx protect cells against H₂O₂-mediated apoptosis, it does not protect against adriamycin-induced apoptosis. They also demonstrated that protective effect of Trx against H₂O₂ is due to enhanced peroxiredoxin expression (6). This study clearly demonstrated that Trx could remove H₂O₂ due to higher Prx activity, but it can not protect against doxorubicin-induced toxicity (6). These results support our data that H₂O₂ does not not play a major role in apoptosis of daunomycin treated Trx overexpressed cells. Therefore, if H₂O₂ were the only cytotoxic compound that mediates doxorubicin-induced apoptosis then probably Trx overexpression would protect against doxorubicin-induced apoptosis. However, the toxicity of anthracyclines predominantly comes from the semiquinone intercalation with the DNA resulting in DNA damage and apoptosis. In our studies we observed that Trx9 cells not only failed to protect against daunomycin-mediated apoptosis, but also enhanced apoptosis in these cells. Thus, data obtained using multiple systems using different approaches confirm a crucial role of Trx in apoptotic response of cancer cells in treatment with daunomycin. Taken together, these data indicate that protective role of Trx could be important in ROS-mediated cytotoxicity. As demonstrated in our data, removal of superoxide anion or H₂O₂ by using MnTBAP decreased p53 DNA binding by about 25-30 percent. Additionally, treating cells with H₂O₂ or other ROS-generating systems did not induce significant p53 DNA binding. This fact demonstrates that ROS are generated due to redox-cycling, but they do not significantly contribute to p53-dependent apoptotic process. Therefore, Trx could inhibit the ROS-mediated cytotoxicity such as that of H₂O₂ as has been shown, but it does not inhibit the redox-cycling process that facilitate the intercalation of the drug with the DNA.

We have earlier shown that Trx induces MnSOD (5). Additionally, Trx does not scavenge O₂⁻ (4). However, it can scavenge hydroxyl radicals or singlet oxygen (4). O₂⁻ is the first reduction product of redox-cycling that could produce H₂O₂, which could be scavenged by Prx or other peroxidases. Therefore, in this circumstance redox-cycling enhancing action of Trx by its electron donating function will remain unaffected. Additionally, our data indicated that NADPH-dependent reductase inhibitor inhibited O₂⁻ generation in Trx9 cells, which suggests that redox-cycling produces enhanced DNA damage in Trx9 cells resulting in p53 activation. These findings are further verified by the fact that dominant-negative expression of redox inactive mutant Trx or silencing of Trx using Trx siRNA failed to induce p53 protein in response to daunomycin suggesting that Trx is required for bioreductive activation of daunomycin.

Our data presented here indicate a fundamental role of endogenous Trx in redox-cycling of anthracyclines, which has not been recognized previously. We have shown previously that E.coli Trx could enhance redox cycling of anthracyclines and induce DNA damage both, in vivo and in vitro (44). We have also shown that E.coli Trx provides reducing equivalents to TR and mammalian cytochrome P450 reductase that enhanced the redox cycling of anthracyclines (44). It is therefore conceivable that Trx may indirectly enhance redox cycling of daunomycin involving NADPH dependant reductase. We also demonstrated that DPIC abolished daunomycin-induced p53 DNA binding, suggesting that redox-cycling of daunomycin depends on NADPH-dependant bioreductive enzymes.

Although daunomycin potently induced p53-DNA binding, other ROS generating agents including menadione did not induce p53-DNA binding (Fig 10B) at concentrations that was used in our study. A previous study has also observed failure of menadione to induce p53–DNA binding in MCF-7 cells (18). However, p53 expression did increase several hours after menadione was removed, indicating that p53 was activated in the DNA damage repair phase (18). Thus, it is unclear why menadione did not activate p53, whereas daunomycin did induce p53 activation. H₂O₂ did not induce a strong p53 response at 500 µM concentration. Increased Trx expression could
have increased H₂O₂ scavenging by peroxiredoxins, which obtain reducing equivalents from Trx. A lower level of p53-DNA binding was observed in Trx mutant Serb4 cells that expresses redox-inactive form of Trx, which does not show higher peroxiredoxin expression (6). This fact suggest that removal of H₂O₂ by overexpression of redox-active Trx in Trx9 cells could not have accounted for less p53 DNA binding in response to H₂O₂. Further, DNA intercalation appears to be essential for induction of p53-DNA binding since 5-iminodaunomycin did induce p53-DNA binding. This fact was further strengthened since there was no involvement of redox cycling in 5-iminodaunomycin, and p53-DNA binding was unaffected in both Trx9 and Serb4 cells.

We also demonstrated decreased O₂⁻ production, p53 expression and apoptosis in daunomycin treated MCF-7 cells deficient in Trx using Trx-siRNA. Accumulating evidence from various studies indicate that p53 is required to enhance chemosensitivity in cancer cells (38,39). In the present study we used MCF-7 cells which are defective in caspase 3, and does not undergo classical 180 bp DNA fragmentation during apoptosis (54), and therefore is dependant on alternate or compensatory mechanisms to induce apoptosis. Further, in p53 defective cells there are alternate regulatory proteins such as Rb or E2F, which could trigger apoptosis. In the present context it becomes more important for cells such as caspase 3 deficient MCF-7 cell lines with wild type p53 to utilize p53 mediated response to induce apoptosis. In summary, our investigation demonstrates a novel pro-oxidant and pro-apoptotic role of Trx in response to anthracycline class of antitumor agents.

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**Figure legends**

**Figure 1. Increased apoptosis of Trx9 cells in response to daunomycin:** (A) Trx clones were treated with daunomycin for 24 hours followed by detection of apoptosis using TUNEL assay kit (Roche Molecular Biochemicals, Indianapolis, Indiana) as described in the methods. Left panel, untreated control cells; right panel, Trx clones treated with daunomycin (1 µM, 24h). FITC panel shows incorporation of Fluorescein dUTP by nick generated in DNA and PI panel shows counter stain with propidium iodide. (B) Bar graph representing percentage of TUNEL positive nuclei in daunomycin treated Trx clones at 24 or 48h. (C) Trx clones were treated with daunomycin (1 µM, 16h) and cell cycle analysis was performed as described in the methods. Left panel, histogram of cell cycle distribution of untreated control cells; right panel, histogram of cell cycle distribution of Trx clones treated with daunomycin. Arrow in right panel indicates “Sub-G1” peak. (D) Data in sub-G1 peak in Fig. 1D is represented as percent apoptotic cells in a bar graph (average of two experiments). (E) Trx activity in MCF-7 clones. Unit of activity is expressed as µmoles of NADPH oxidized/min/mg protein.

**Figure 2. Enhanced clonogenic death of MCF-7 cells and A549 cells overexpressing redox-active Trx, but not redox-inactive Trx. (A)** Vector, serb4 or Trx9 cells were trypsinized and seeded to a final density of 1 X 10⁶ viable cells per100 mm² dishes and allowed to attach overnight. Cells were then treated with appropriate concentration of drugs for 24 hours, trypsinized and seeded to a final density of 500,000 viable cells per 100 mm² dish. Viable cells were determined using Vicell counter (Beckman Coulter). After 14 days, the surviving colonies were washed in PBS, fixed in 70% ethanol and stained using 0.1% crystal violet in 90% ethanol. (B). Stable clones of A549 cells expressing Trx (Trx) or redox-inactive Trx (dnTrx) were processed as described for Fig 2A.

**Figure 3. Silencing Trx expression by RNAi decreases apoptosis in MCF-7 cells in response to anthracyclines. (A)** Transfection of Trx-specific RNAi inhibits Trx expression in MCF-7 cells. MCF-7 cells were transfected with indicated concentrations of non-targeting or Trx siRNA as described in methods. Expression of Trx was determined by western blot as described in methods. β-actin is shown as loading control. (B) Trx siRNA down regulates expression of p53, active caspase-7 and cleaved PARP expression in response to daunomycin. Non-targeting siRNA or Trx siRNA transfected MCF-7 cells were treated with 1 µM daunomycin for 16 hours. Western analysis was performed for the detection of p53, caspase 7 and cleaved PARP as described in the method. Lanes 1-3, Control cells transfected with non targeting siRNA in triplicates; lanes 4-6, untreated Trx siRNA transfected cells in triplicates; Lanes, 7-9, cells transfected with non-targeting siRNA and treated with 1 µM daunomycin (16h); lanes 10-12, cells transfected with Trx siRNA and treated with 1 µM daunomycin (1 µM). β-actin is shown as loading control. (C) Ratio of p53 level (upper panel of Fig 3B) to β-actin level. ** Significantly less compared to p53 level in NT transfected and daunomycin-treated cells in Fig 3B. (D). Ratio of caspase-7 level (middle panel of Fig 3B) to β-actin level. ** Significantly less compared to caspase-7
level in NT (Non-targeting) transfected and daunomycin-treated cells in Fig 3B. (E). Ratio of cleaved PARP level (lower panel of Fig 3B) to β-actin level. ** Significantly less compared to p53 level in NT transfected and daunomycin-treated cells in Fig 3B.

**Fig 3F. Effect of Trx silencing on caspases-1, -6, and -8 expression in response to daunomycin.** MCF-7 cells were treated with Trx siRNA or NT siRNA, and treated with daunomycin (1 µM) for 16 hours. Immunoblotting was performed for caspases-1, caspases-6 and cleaved caspases-8 using respective specific antibodies. Upper panel, caspases-1; middle panel, caspases-6; lower panel, caspases-8; lowest panel, β-actin. Lanes, 1-3 cells transfected with NT siRNA; lanes 4-6, cells transfected with Trx siRNA; lanes 7-9, cells treated with NT siRNA and treated with daunomycin; lanes 10-12, cells treated with Trx siRNA and treated with daunomycin. Fig 3G. Densitometry of caspases-1 western blotting.

**Figure 4. Silencing Trx expression by RNAi decreases expression of p53 in response to anthracyclines in A549 cells. (A). Transfection of Trx-specific RNAi inhibits Trx expression in A549 cells.** A549 cells were transfected with indicated concentrations of non-targeting or Trx siRNA as described in methods. Expression of Trx was determined by western blot as described in methods. β-actin is shown as loading control. (B) Trx siRNA down regulates expression of p53 in response to daunomycin. Non-targeting siRNA or Trx siRNA transfected A549 cells were treated with 1 µM daunomycin for 16 hours. Western analysis was performed for the detection of p53 as described in the method. Lanes 1-3, Control cells transfected with non-targeting siRNA in triplicates; lanes 4-6, untreated Trx siRNA transfected cells in triplicates; Lanes, 7-9, cells transfected with non-targeting siRNA and treated with 1 µM daunomycin (16h); lanes 10-12, cells transfected with Trx siRNA and treated with 1 µM daunomycin (1 µM). β-actin is shown as loading control. (C) Ratio of p53 level (upper panel of Fig 4A) to β-actin level** ** Significantly less compared to p53 level in NT transfected and daunomycin-treated cells in Fig 4A.

**Figure 5. Effect of Trx overexpression on daunomycin-induced p53 expression:** (A) Trx clones were treated with 1 µM daunomycin for 16 hours followed by lysate preparation as described in the method. p53 or phospho-p53 (ser-15) was detected as described in the method. Lanes 1-3, untreated control cells; lanes 4-6, Trx clones treated with daunomycin. Upper panel, p53; middle panel, p53 (ser-15); lower panel, loading control (β-actin). (B) Stable clones of MCF-7 expressing redox-active Trx or redox-inactive Trx (dnTrx) were generated by transfecting cells with pCMV-Trx or pCMV-dnTrx constructs and selecting clones using G418, 800µg/ml. Expression of Trx was determined by western analysis as described previously. (C). MCF-7 clones were treated with 1 µM daunomycin for 16 hours, followed by p53 western analysis as described previously. Lower panel, β-actin of same blot. (D) Overexpression of Trx or dnTrx in MCF-7 cells using adenovirus infection. MCF7 cells were infected with AdenoX LacZ, AdenoX-Trx or AdenoX-dnTrx, and Trx was determined using an ELISA assay as described (30). The amount of Trx was expressed as picograms of Trx per mg protein. (E) MCF-7 cells were infected with adenovirus (AdenoX LacZ, AdenoX Trx or Adx dnTrx), and p53 was determined using an ELISA assay as described (30). (F). MCF-7 cells were infected with adenovirus as described in the methods section. Cells were treated with 1 µM daunomycin after 48h, and following 16 hours of incubation with the drug western analysis was performed for p53 and β-actin as described in the methods. (G)
clones cells treated with daunomycin. (B) Effect of adenovirus-mediated overexpression of thioredoxin in daunomycin induced p53-DNA binding: MCF-7 cells were infected with adenovirus (AdenoX LacZ, AdenoX Trx or AdenoX dnTrx) as described in the method. After 48 hours infected cells were treated with 1 µM daunomycin (4h) and nuclear extract was prepared and gel-shift assay was performed as described in the method. Lanes 1-3, untreated control cells; lanes 4-6, daunomycin treated cells.

Figure 7. Effect of p53 down regulation using RNA interference on apoptosis in MCF-7 cells. Non-targeting siRNA or p53siRNA transfected MCF-7 cells were treated with 1 µM daunomycin for 16 hours. Western analysis was performed for the detection of p53, caspase 7 and PARP as described in the method. Lanes 1-6, Control cells transfected with non-targeting or Trx-siRNA in triplicates, lanes 7-12, Non-targeting siRNA or Trx-siRNA transfected MCF-7 cells were treated with 1 µM daunomycin in triplicates. b-actin is shown as loading control. (B) Ratio of p53 level (upper panel of Fig 7A) to b-actin level. ** Significantly less compared to p53 level in NT transfected and daunomycin-treated cells in Fig 7A. (C) Ratio of caspase-7 level (2nd panel of Fig 7A) to b-actin level. **Significantly less compared to caspase-7 level in NT transfected and daunomycin-treated cells in Fig 7A. (D) Ratio of cleaved PARP level (upper panel of Fig 7A) to b-actin level. ** Significantly less compared to cleaved PARP level in NT transfected and daunomycin-treated cells in Fig 7A.

Figure 8. Generation of ROS in Trx clones in response to daunomycin. (A) Trx clones were treated with daunomycin (1 µM) or 5-imidodaunomycin (1 µM) and processed for DCF-DA assay as described in the method. Left panel, untreated control cells; middle panel, cells treated with daunomycin; right panel, cells treated with 5-imidodaunomycin; Inset, mid section of confocal microscopy scan showing most of the fluorescence localized in the cytosol. (B) Graph showing change in intensity of DCF-DA fluorescence over time indicated in seconds. (C) Control and 5-imidodaunomycin treated Trx clones. (D) Generation superoxide anion in Trx clones in response to daunomycin. Trx clones were treated with daunomycin and the generation of O$_2^-$ was determined by reduction of SOD-inhibitable cytochrome c as described in the method. Data was presented as nmole of O$_2^-$ produced per mg total cell protein. (E) Effect of adenovirus-mediated overexpression of thioredoxin on redox cycling of daunomycin. Adenovirus-mediated Trx transfected MCF-7 cells were treated with daunomycin and the generation of O$_2^-$ was determined by reduction of SOD-inhibitable cytochrome c as described in the method. Data was presented as nmole of O$_2^-$ produced per mg total cell protein. (F) Trx siRNA decreases redox cycling of daunomycin. Non-targeting or Trx siRNA transfected MCF-7 cells were treated with daunomycin and the generation of O$_2^-$ was determined by reduction of SOD-inhibitable cytochrome c as described in the method. Data was presented as nmole of O$_2^-$ produced per mg total cell protein. (G) Effect of silencing Trx on daunomycin redox cycling in A549 cells. A549 cells were transfected with NT or Trx siRNA, and O$_2^-$ generation was determined as described for Fig 8F.

Figure 9. Effect of reductase inhibitor DPIC on O$_2^-$ generation: Trx9 cells were treated with DPIC as indicated in the Figure, and O$_2^-$ generation was assayed as described in the method. Lane 1, Trx9 cells treated with 10 µM daunomycin. The generation of O$_2^-$ in this treatment was considered 100%. Lane 2, Trx9 cells pre-incubated with 25 µM DPIC followed with 10 µM daunomycin treatment; lane 3, Trx9 cells pre-incubated with 50 µM DPIC followed with 10 µM daunomycin treatment.

Figure 10. (A) Effect of 5-Iminodaunomycin and ROS generating agents on p53-DNA binding. Trx clones were treated with indicated concentration of superoxide-generating agents for 4-hr. Following incubation nuclear extract was prepared and p53 EMSA was performed as described. (B) Trx9 cells were treated with indicated concentration of superoxide-generating agents for 4-hr. Following incubation nuclear extract was prepared and p53 EMSA was performed as described in the methods. (C) Effect of MnTBAP and DPIC on daunomycin-induced p53 DNA binding in Trx9 cells: Trx9 cells were treated with daunomycin either in presence of
MnTBAP (20 μM) or DPIC (50 μM) for 4 hours and gel-shift assay was performed as described in the methods. Lane 1, untreated Trx9 cells; lane 2, Trx9 cells treated with daunomycin (1 μM); lane 3, Trx 9 cells treated with 100 μM MnTBAP; lane 4, Trx9 cells treated with daunomycin + MnTBAP; lane 5, Trx9 cells treated with DPIC; lane 6, Trx9 cells treated with daunomycin + DPIC. Lower panel; expression of phospho-p53-Ser15 in response to daunomycin. Trx9 cells were exposed to various treatments as described for the upper panel. The level of phospho-p53-Ser15 was determined by western analysis as described in the methods section.
Figure 1A

Figure 1B

Percent control TUNEL positive cells

24 Hours 48 Hours

Vector  Trx 9  Serb 4  Vector  Trx 9  Serb 4

***  ***  ***
Figure 1C
Figure 1D

Percent Apoptotic Cells

Vector  Trx9  Serb4

Figure 1E

µmoles of NADPH oxidized/min/mg Protein

Vector  Trx9  Serb4
Figure 2A

![Bar graph showing the number of colonies per 100mm dish for different conditions.](image)

**X-axis:** Daunomycin concentration (0.025µM and 0.05µM)
**Y-axis:** Number of colonies/100mm dish

Legend:
- Vector
- Serb4
- Trx9

Control: 0.1µM Daunomycin

Figure 2B

![Image showing colony growth under different conditions.](image)
**Figure 3A**

| MCF-7 siRNA (nM) | Control | 30 | 100 | 30 | 60 | 100 |
|------------------|---------|----|-----|----|----|-----|
| Non-Target       | Thiorodoxin | β-actin |
| Trx siRNA        |          |      |     |     |     |     |

**Figure 3B**

| Control | Daunomycin |
|---------|------------|
| Non target siRNA | Non target siRNA |
| Trx siRNA      | Trx siRNA    |
|                | p53          |
|                | Active caspase-7 |
|                | Cleaved PARP |
|                | β-actin      |
Figure 3C

Densitometry of p53 normalized to β-actin

Control Daunomycin

NT siRNA Trx siRNA

**
Figure 3D

Densitometry of active caspase-7 normalized to β-actin

- NT siRNA
- Trx siRNA

Control
Daunomycin

Figure 3E

Densitometry of cleaved PARP normalized to β-actin

Control
Daunomycin
Figure 3F

![Western Blot Image]

| NT siRNA | Trx siRNA | NT siRNA + Daun | Trx siRNA + Daun |
|----------|-----------|-----------------|-----------------|
| Caspase-1|
| caspase-6|
| caspase-8|
| β-actin  |

Figure 3G

![Bar Graph Image]

Densitometry of Caspase-1 normalized to β-actin

- Control
- Daunomycin

- NT siRNA
- Trx siRNA
**Figure 4A**

Trx siRNA

| A549 | 40nM | 50nM | 60nM |
|------|------|------|------|
| Trx  |      |      |      |
| β-actin | | | |

**Figure 4B**

Control | Daunomycin

| A549 | Trx siRNA | A549 | Trx siRNA |
|------|-----------|------|-----------|
| p53  |           | p53  |           |
| β-actin |         | β-actin |         |

**Figure 4C**

Densitometry of p53, normalized to β-actin

- NT siRNA
- Trx siRNA

**Control**

- 0.15 ± 0.02

**Daunomycin**

- 0.70 ± 0.03

**p** < 0.01
Figure 5A
Figure 5B

Figure 5C
Figure 5D

![Graph showing the expression levels of p53 and β-actin in different conditions.]

Figure 5E

![Western blot analysis showing the expression levels of p53 and β-actin.]

- Adx-LacZ
- Adx-Trx
- Adx-dnTrx

Picolgrams of Thioredoxin per protein
Figure 5F

|        | pCMV-dnTrx | pCMV-Trx | pCMV |
|--------|------------|----------|-------|
| Control| -          | -        | +     |
| Daunomycin, 1µM | -          | -        | +     |

**Western Blot Analysis**

- **p53**
  - Control: +
  - Daunomycin, 1µM: +

- **β-actin**
  - Control: +
  - Daunomycin, 1µM: +
Figure 6A

| Control  | Daunomycin |
|----------|------------|
| Vector   | Serb4, Trx9|
|          | vector, Serb4, Trx9 |

p53-DNA complex

Figure 6B

Adx LacZ
Adx Trx
Adx dnTrx

Adx LacZ
Adx Trx
Adx dnTrx

p53-DNA complex
Figure 7A

Figure 7B
Figure 7C

Densitometry of active caspase-7 normalized to β-actin

Control Daunomycin

NTsiRNA p53 siRNA

Figure 7D

Densitometry of cleaved PARP normalized to β-actin

Control Daunomycin

NTsiRNA p53 siRNA
Figure 8A

![Image of Figure 8A showing samples under different conditions: CONTROL, DAUNOMYCIN, and 5-IMINODAUNOMYCIN for different groups: Vector, Trx9, and Serb4. The images show fluorescence staining results.](http://www.jbc.org/Downloaded from)
Figure 8B
Figure 8C

DCF-DA fluorescence intensity vs. time in seconds for different treatments:
- Serb4 - 5iminodan
- Vec-5iminodan
- TRX-5iminodan
- TRX
- Serb-4
- Vec
- CONTROL
Figure 8D

nM superoxide produced / min / mg protein

Vector
Trx-9
Serb -4

Control  Daunomycin  5-iminodaunorubicin
Figure 8E
Figure 8F

![Graph showing nmoles of superoxide produced/min/mg protein for NT siRNA and Trx siRNA with Daunomycin as the x-axis](image-url)
Figure 8G

The figure shows a bar graph comparing nM superoxide produced/min/mg protein for different conditions:

- **A549**
- **A549 + Trx siRNA**
- **A549 + Daunomycin**

The graph includes two conditions:

- **Blank**
- **Daunomycin**

The y-axis represents nM superoxide produced/min/mg protein, ranging from 0 to 25.
### Figure 10A

| Control | 5-imino Daunomycin | H$_2$O$_2$ | Menadione | Taxol |
|---------|-------------------|-----------|-----------|-------|
| Vector  |                   |           |           |       |
| Tx.9    |                   |           |           |       |
| Serb.4  |                   |           |           |       |

**p53-DNA complex**

### Figure 10B

| Control | Daunomycin 1μM | Pyocyanine 2.5μM | Menadione 20μM | PMS 20μM | H$_2$O$_2$ 500μM |
|---------|----------------|------------------|----------------|----------|-----------------|
| Vector  |                |                  |                |          |                 |
| Tx.9    |                |                  |                |          |                 |
| Serb.4  |                |                  |                |          |                 |

**p53-DNA complex**
Figure 10C

- **p53-DNA complex**
- **Phospho-p53**
Endogenous thioredoxin is required for redox-cycling of anthracyclines and p53-dependent apoptosis in cancer cells
Dashnamoorthy Ravi, Harish Muniyappa and Kumuda C. Das

*J. Biol. Chem.* published online September 13, 2005

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