

## Cellular Levels of Thioredoxin Associated with Drug Sensitivity to Cisplatin, Mitomycin C, Doxorubicin, and Etoposide<sup>1</sup>

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### Abstract

Thioredoxin, a cellular thiol, functions as a self-defense mechanism in response to environmental stimuli, including oxidative stress. We first determined cellular levels of thioredoxin in several human bladder and prostatic cancer cell lines resistant to *cis*-diamminedichloroplatinum(II) (cisplatin). All cisplatin-resistant cell lines had much higher levels of thioredoxin than those in their drug-sensitive parental counterpart. We then, by introducing thioredoxin antisense expression plasmids into human bladder cancer T24 cells, established two bladder cancer cell lines that had decreased levels of thioredoxin. These thioredoxin antisense transfectants showed increased sensitivity to cisplatin and also to other superoxide-generating agents, *i.e.*, doxorubicin, mitomycin C, etoposide, and hydrogen peroxide, as well as to UV irradiation, but not to the tubulin-targeting agents, vincristine, and colchicine. Cellular levels of thioredoxin thus appear to limit sensitivity to various superoxide-generating anticancer drugs in cancer cells.

### Introduction

Cisplatin,<sup>3</sup> a potent antitumor agent, has been widely used for the treatment of many malignancies, including testicular, ovarian, head and neck, bladder, esophageal, and small lung cancers (1). However, the development of cisplatin resistance in these tumors is an obstacle in cancer chemotherapy (2). Clarification of the mechanisms whereby cisplatin resistance occurs is required to improve the therapeutic effects of these agents. Pleiotropic mechanisms responsible for cisplatin resistance include a decrease in drug accumulation and an increase in drug detoxification by thiol-containing molecules, such as glutathione and metallothionein, and the repair of DNA damage or DNA topoisomerase I (1-5).

Thioredoxin, a cellular thiol, as are glutathione and metallothionein, has been implicated in the regulation of some redox-regulated molecules, including NF- $\kappa$ B, AP-1, and glucocorticoid receptor (6-9). Thioredoxin also has other multiple functions, such as radical-scavenging action, signal transduction, and defense reaction to hydrogen peroxide and tumor necrosis factor (10). To determine whether changes in redox potential modulate sensitivity to cisplatin in human cancer cells, we first examined whether thioredoxin levels were altered in cisplatin-resistant cancer cells and next examined whether cellular sensitivity to cisplatin or other superoxide-generating agents was altered in cancer cell lines carrying thioredoxin antisense expres-

sion plasmids. Our experimental results demonstrated a possible association of thioredoxin levels with drug resistance to cisplatin and other superoxide-generating agents, *i.e.*, mitomycin C, doxorubicin, etoposide, and hydrogen peroxide, as well as UV light irradiation.

### Materials and Methods

**Materials.** Cisplatin was donated by Bristol Myers Co. (Kanagawa, Japan). Doxorubicin, vincristine, and colchicine were from Sigma Chemical Co. (St. Louis, MO). Etoposide and mitomycin C were obtained from Nippon Kayaku Co. (Tokyo, Japan) and Kyowa Co. (Tokyo, Japan), respectively. Hybond N<sup>+</sup> membranes, nitrocellulose membrane, and the DNA labeling kit were obtained from Amersham International, and [ $\alpha$ -<sup>32</sup>P]dCTP was from Dupont New England Nuclear. Thioredoxin cDNA (11) and glyceraldehyde-3-phosphate dehydrogenase cDNA (12) were used for Northern blot analysis. Lipofectin was purchased from BRL (Bethesda, MD). G418 was purchased from GIBCO/BRL.

**Cell Culture and Cell Lines.** We used several cisplatin-resistant cell lines: P/CDP4 and P/CDP5, derived from PC-3 cells (3), and 5R-5 have been reported previously (3); T24/DDP5, T24/DDP7, and T24/DDP10 derived from human bladder cancer T24 cells (5); and KK47/CB30 and KK47/CB60 derived from human bladder cancer KK47 cells.<sup>4</sup> Two cisplatin-resistant cell lines, KK47/CB30 and KK47/CB60, were established after continuous exposure to increasing doses of the drug, as described in our previous study (5). These cell lines were cultured in Eagle's MEM (Nissui Seiyaku Co., Tokyo, Japan) containing 10% FCS (Sera-Lab, Ltd., Sussex, England), 1 mg/ml Bactopectone (Difco Laboratories, Detroit, MI), 0.292 mg glutamine/ml, 100  $\mu$ g/ml kanamycin, and 100 units/ml penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Colony Formation Assay.** We seeded 400-1000 cells in 35-mm dishes in the absence of drugs at 37°C for 18 h. For treatment with hydrogen peroxide and mitomycin C, the cells were washed with PBS and exposed to drugs in serum-free medium for 1 h at 37°C, after which the medium was replaced with fresh medium. For UV irradiation, the cells were seeded in 60-mm dishes and washed with PBS the next day; they were then aspirated before being irradiated with UV. Following the irradiation, fresh growth medium was added, and the cells were then incubated for 6 days. For the other drugs, the cells were continuously incubated for an additional 6 days with the drugs, as described previously (3, 5).

**Western Blot Analysis.** Anti-GST- $\pi$  blotting was performed as described previously (13). For thioredoxin, 30  $\mu$ g of whole-cell lysates were analyzed by 15% SDS-PAGE. Protein fractions from the gel were transferred onto a nitrocellulose membrane in 25 mM Tris-HCl (pH 8.3)-92 mM glycine-20% methanol for 2 h at 200 V. The membranes were then incubated with antiserum against human thioredoxin (1:400) for 1 h at room temperature. Anti-human thioredoxin antibody was prepared as described previously (14). Detection was performed with enhanced chemoluminescence Western blotting detection reagents (Amersham, Buckinghamshire, England).

**Northern Blot Analysis.** Total RNA was isolated with guanidine isothiocyanate, and Northern blot analysis was performed as described previously (5). Radioactivity was detected with a Fujix Bas 2000 bio-imaging analyzer (Fuji Photo Film Co., Tokyo, Japan) (12).

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<sup>3</sup> The abbreviations used are: cisplatin, *cis*-diamminedichloroplatinum(II); GST- $\pi$ , glutathione thiol transferase- $\pi$ .

**Construction of Antisense Thioredoxin Expression Vector.** Plasmid SR $\alpha$ ADF (11) and mammalian expression vector pRC/CMV (Invitrogen Corp., San Diego, CA) were used to construct an antisense thioredoxin expression vector. SR $\alpha$ ADF was digested with *Bam*HI and *Hind*III, and about 340 bp cDNA from the open reading frame of human thioredoxin was isolated. The fragment was subcloned into the *Hind*III sites of the pRC/CMV after *Hind*III linker ligation and transformed into competent *Escherichia coli*. Mini-preparations of ampicillin-resistant clones were analyzed for the orientation of the inserts, as determined by restriction site mapping.

**Transfection with Antisense Thioredoxin Expression Vector.** Exponentially growing T24 cells ( $5 \times 10^6$ ) were washed with PBS, and the medium was replaced with serum-free medium. A mixture of 50  $\mu$ g Lipofectin and 10  $\mu$ g antisense thioredoxin expression vector DNA was then added. After 12 h, the medium containing DNA and Lipofectin was replaced with fresh medium. The cells were then incubated in selection medium containing 400  $\mu$ g/ml geneticin (G418) for 3 to 4 weeks. G418-resistant colonies were cloned in the presence of G418, and transfectants with reduced thioredoxin expression were further selected. Two stable transfectants, AS19 and AS22, which showed reduced thioredoxin expression, were selected from among 32 G418-resistant clones. We also used two G418-resistant clones, AS3 and AS23, which had levels of thioredoxin similar to those in the parental T24 cells. These transfectants were continuously cultured in the presence of 400  $\mu$ g/ml G418 (13).

## Results and Discussion

The dose-response curves of cisplatin-resistant cell lines and their parental counterparts (PC-3, T24, and KK47) to various drugs and to UV irradiation were determined by colony formation assay. The relative resistance and IC<sub>50</sub> of each parental cell line are shown in Table 1. The relative resistance in the cisplatin-resistant cell lines derived from T24 and PC-3 was similar to that reported previously (3, 5). Northern blot analysis revealed increased levels of thioredoxin mRNA in all cisplatin-resistant cell lines compared with levels in their parental counterparts, T24, KK47, and PC-3 (Fig. 1A). Cellular thioredoxin mRNA levels were 4- and 5.8-fold higher in P/CDP4 and P/CDP5, respectively, than in the parental PC-3 cells, while thioredoxin mRNA level in a drug-sensitive revertant of P/CDP5, 5R-5, was similar to that in PC-3 (Fig. 1A; Table 1). In comparison with the cisplatin-resistant cell lines derived from T24 or KK47, two prostatic cancer cell lines resistant to cisplatin had much higher thioredoxin mRNA levels than PC-3 cells (Fig. 1A; Table 1). Western blot analysis also demonstrated that the cellular content of thioredoxin was much higher in the cisplatin-resistant variants than in their parental counterparts (Fig. 1B).

To determine whether cellular sensitivity to cisplatin or other superoxide-generating agents was closely associated with cellular levels of thioredoxin, we established T24 sublines that had decreased thioredoxin levels by introducing thioredoxin antisense expression

plasmids (see "Materials and Methods"). Two G418-resistant cell lines, AS19 and AS22, had decreased levels of thioredoxin, and two G418-resistant cell lines, AS3 and AS23, had levels of thioredoxin similar to those in T24 cells (Fig. 2). The thioredoxin levels in AS19 and AS22 cells were 0.3- and 0.5-fold, respectively, those in T24 or AS3 and AS23. By contrast, AS3, AS19, AS22, and AS23 cells had levels of GST- $\pi$  similar to those in the parental T24 cells (Fig. 2).

The dose-response curves of the four transfectants, AS3, AS19, AS22, and AS23, to cisplatin demonstrated that AS19 and AS22 were 2- to 3-fold more sensitive to the cytotoxic effect of cisplatin than AS3 and AS23 (Fig. 3). Both AS3 and AS23 showed sensitivity to cisplatin similar to that of their parental T24 cells. We also examined whether AS19 and AS22 showed altered sensitivity to other drugs as well as UV light irradiation. AS19 and AS22 also showed increased sensitivity to doxorubicin, mitomycin C, etoposide, hydrogen peroxide, and UV in comparison with AS3 and AS23 (Table 2). However, the sensitivity of AS19 and AS22 to vincristine and colchicine was similar to that of AS3 and AS23.

In this study, we first demonstrated that almost all the cisplatin-resistant cell lines derived from human bladder cancer (KK47 and T24) and prostatic cancer (PC-3) cells had increased expression of thioredoxin. The second assay, with the thioredoxin antisense expression plasmid, demonstrated a reduction of thioredoxin levels associated with an increase in sensitivity to cisplatin. These two independent experiments indicate a possible association of thioredoxin levels with sensitivity to cisplatin in human bladder or prostatic cancer cells. The acquisition of cisplatin resistance appears to be mediated through pleiotropic mechanisms (1, 2). The reduced accumulation of cisplatin in P/CDP4 and P/CDP5 cells is possibly mediated through the increased ATP-dependent efflux activity of the drug (Refs. 3 and 15; Table 1). T24/DDP5, T24/DDP7, and T24/DDP10, which have much higher DNA topoisomerase I levels than T24, showed collateral sensitivity to a topoisomerase I-targeting camptothecin derivative, (4*s*)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]dione hydrochloride trihydrate (CPT-11; Ref. 5; Table 1). The cisplatin-resistant cell lines, KK47/CB30 and KK47/CB60, derived from human bladder cancer KK47 cells, showed reduced drug accumulation, as well as increased expression of metallothionein,  $\gamma$ -glutamyl-cysteine synthetase, and GST- $\pi$ <sup>4</sup> (Table 1). Although the mechanism of resistance to cisplatin in the variants derived from the three different parental counterparts appeared to differ (Table 1), all of these variants had increased thioredoxin levels, suggesting the involvement of a common mechanism, *i.e.*, redox regulation, produced by this thioredoxin.

Drug resistance to cisplatin or alkylating agents is often influenced

Table 1 Human cell lines resistant to cisplatin

Cell line	Parental cell line	Relative resistance <sup>a</sup>	Thioredoxin expression <sup>b</sup>	Derivation
T24		1.0	1.0	Human bladder cancer cell line <sup>c</sup>
T24/DDP5	T24	2.2	1.6	Overexpression of Topo I
T24/DDP7	T24	5.2	2.1	Overexpression of Topo I
T24/DDP10	T24	8.4	3.1	Overexpression of Topo I, decreased cisplatin accumulation
KK47		1.0	1.0	Human bladder cancer cell line <sup>d</sup>
KK47/CB30	KK47	9.4	1.9	Overexpression of GST- $\pi$ and metallothionein
KK47/CB60	KK47	19.5	3.0	Overexpression of GST- $\pi$ and metallothionein
PC-3		1.0	1.0	Human prostatic cancer cell line <sup>e</sup>
P/CDP4	PC-3	11.0	4.0	Decreased cisplatin accumulation
P/CDP5	PC-3	23.0	5.8	Decreased cisplatin accumulation
5R-5	P/CDP5	0.9	1.1	Drug-sensitive revertant of P/CDP5

<sup>a</sup> Relative resistance to cisplatin was calculated as IC<sub>50</sub> of drug-resistant cell line divided by IC<sub>50</sub> of each parental cell line, IC<sub>50</sub> being the drug dose required to reduce the initial survival fraction to 50%.

<sup>b</sup> Calculated on the radioactivity from Northern blot analysis, normalized by those of glyceraldehyde-3-phosphate dehydrogenase (see Fig. 1).

<sup>c</sup> The isolation and properties of cisplatin-resistant cell lines derived from T24 have been described previously (5). Topo I, DNA topoisomerase I.

<sup>d</sup> The properties of cisplatin-resistant cell lines derived from KK47 are to be published elsewhere<sup>4</sup> (also see "Materials and Methods").

<sup>e</sup> The cisplatin-resistant cell lines derived from PC-3 have been characterized previously (3).

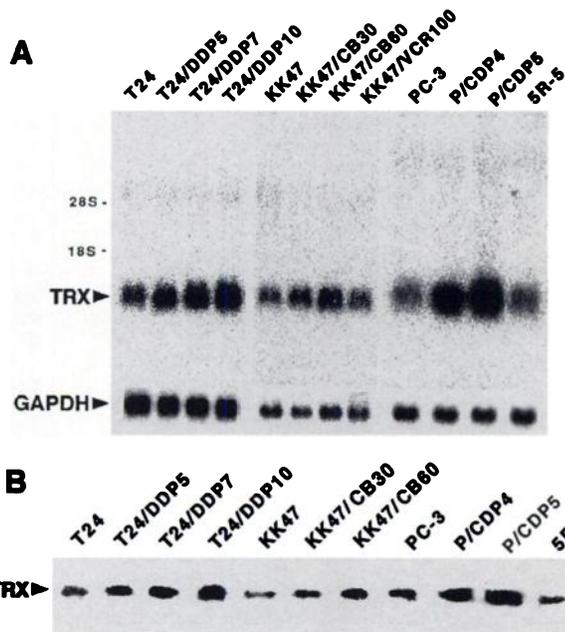


Fig. 1. Northern (A) and Western (B) blot analysis for thioredoxin (TRX) in the T24, KK47, and PC-3 parental cell lines and their cisplatin-resistant derivatives. A, total RNA (15 µg) extracted from each cell line was resolved by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde, transferred to Hybond N<sup>+</sup>, and hybridized with <sup>32</sup>P-labeled thioredoxin cDNA probes. The equivalent loading of total RNA is shown by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) blot. B, whole-cell lysates (30 µg) from each cell line were resolved on 15% SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with rabbit antibody against human thioredoxin and horseradish peroxidase conjugated anti-rabbit IgG.

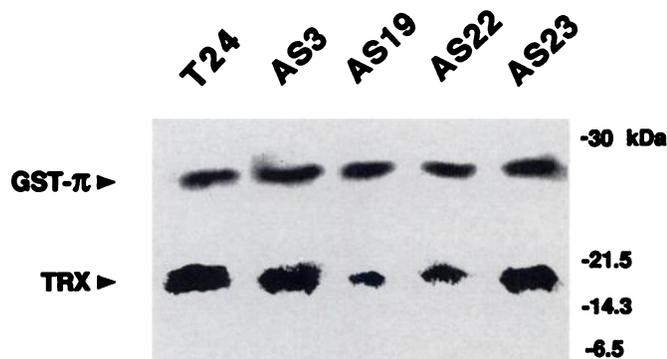


Fig. 2. Western blot analysis of thioredoxin (TRX) and GST-π in T24 cells stably transfected with antisense thioredoxin expression vectors. Thirty µg and 100 µg of cell lysates were used for Western blot analysis of thioredoxin and GST-π, respectively.

by intracellular thiols (1, 2, 12). Indeed, one could argue that thioredoxin has a protective role, functioning against the environmental insults of cisplatin or other agents. Thioredoxin is an intracellular thiol, as are glutathione and metallothionein. Cisplatin has high affinity to thiols (SH; Refs. 1 and 2), and we would expect thioredoxin to react with cisplatin via its dithiols in the cytosol fraction of cancer cells. Masuda *et al.* (16) have recently reported that cisplatin produces superoxide anions when it reacts with its target DNA in a cell-free system. Gergel *et al.* (17) have reported that cisplatin increased lipid peroxidation in kidney homogenate, suggesting that the effect of cisplatin on lipid peroxidation may be linked with its nephrotoxicity. The superoxide anions show cytotoxicity to growing cells, and thioredoxin protects the cells from oxidative stress (10, 14). Altered levels of thioredoxin in the cisplatin-resistant variants, and also in the

stable antisense transfectants, could modulate superoxide-induced cytotoxicity, resulting in altered sensitivity to cisplatin. Thioredoxin also influences gene expression through the activation of NF-κB (7) and enhances Jun/Fos binding to AP-1 site in the presence of thioredoxin (8). Some cisplatin-resistant cell lines preferentially repair cisplatin interstrand cross-links in transcriptionally active genes (1). It is, therefore, possible that thioredoxin may thus activate the transcription factor(s) of DNA repair enzymes or that it may repair associated protein(s), resulting in altered sensitivity to cisplatin and other agents that react with DNA.

The reduced expression of thioredoxin also sensitized bladder cancer T24 cells to doxorubicin, mitomycin C, etoposide, hydrogen peroxide, and UV irradiation (Table 2). Both doxorubicin and mitomycin C break DNA strands, this being induced by oxygen radicals produced by redox cycling through the semiquinone intermediate

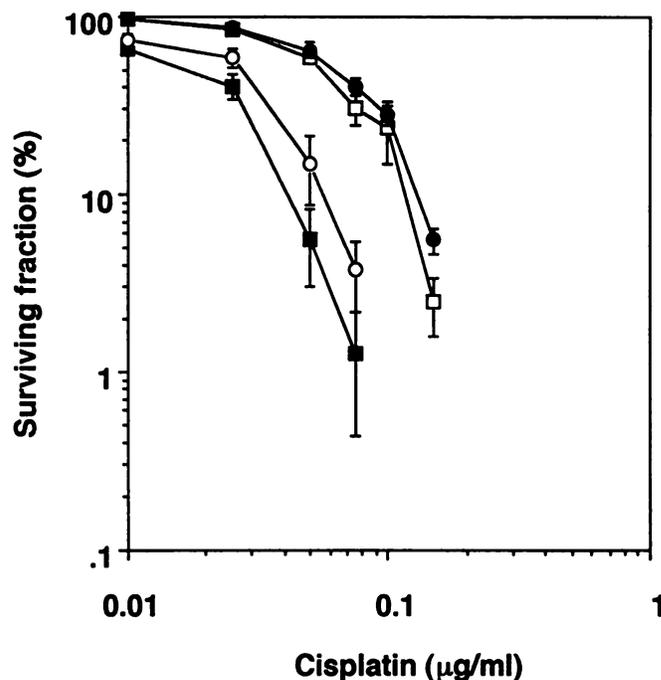


Fig. 3. Dose-response curves to cisplatin of T24 sublines transfected with antisense thioredoxin expression plasmids. Four hundred AS3 cells (□) and AS23 cells (●), 600 AS19 cells (■), and AS22 cells (○) were seeded and incubated in the absence of any drugs for 18 h. The cells were then exposed to various concentrations of cisplatin for 7 days. The number of colonies was counted after Giemsa staining; 100% corresponds to colony number of each cell line in the absence of any drug. Each point indicates the average value for triplicate dishes. Bars, SD.

Table 2. Sensitivity of T24 transfectants to various drugs and to UV<sup>a</sup>

Drug	IC <sub>50</sub> (nM) AS3	Relative resistance			
		AS19	AS22	AS23	
Doxorubicin	4.1	0.4	0.6	1.0	
Mitomycin C	170	0.4	0.5	1.1	
Etoposide	25	0.7	0.7	1.0	
Vincristine	3.0	1.0	0.9	1.0	
Colchicine	0.96	0.9	1.1	1.0	
H <sub>2</sub> O <sub>2</sub>	420	0.4	0.5	1.0	
UV	5.1 <sup>b</sup>	0.5	0.7	1.1	

<sup>a</sup> The sensitivity of each cell line was determined from the dose-response curve as described in "Materials and Methods." The IC<sub>50</sub> of AS3 for each drug is expressed; the mean IC<sub>50</sub> was obtained from two separate experiments, with duplicate dishes. Relative resistance was obtained by dividing the mean IC<sub>50</sub> of the three cell lines by that of the AS3 cells.

<sup>b</sup> J/m<sup>2</sup>.

(18). The cytotoxicity of etoposide is due to the formation of DNA-drug-topoisomerase II cleavable complexes (19). Etoposide is oxidized into an active metabolite, orthoquinone, which is induced via the semiquinone free radical (19, 20). In addition, UV irradiation also induces free radicals and oxygen radicals (21). Doxorubicin, mitomycin C, etoposide, hydrogen peroxide, and UV irradiation are thought to induce oxidative stress, and cellular sensitivity to these agents could thus be influenced by the decreased expression of thioredoxin. However, the decreased expression of thioredoxin brought about no changes in the cellular sensitivity to the two tubulin-targeting agents, vincristine and colchicine (Table 2). In conclusion, we demonstrated that cellular levels of thioredoxin were closely associated, not only with intracellular oxidative stress, but also with the cytotoxic stress brought about by cisplatin and other superoxide-generating agents. These findings suggest that thioredoxin could constitute an immediate SOS signal for various environmental stimuli, including anticancer agents.

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