

# Synergistic effect of peroxiredoxin II antisense on cisplatin-induced cell death

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Abbreviations: Prx II, peroxiredoxin II; CI, combination index

## Abstract

**Peroxiredoxin II (Prx II) is known not only to protect cells from oxidative damage caused by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), but also to endow cancer cells with resistance to both H<sub>2</sub>O<sub>2</sub> and cisplatin and to grant them radioresistance. In this study, we examined whether Prx II antisense could enhance cisplatin-induced cell death. When gastric cancer cells were transfected with various concentrations of Prx II antisense plasmid, pPrxII/AS, and then treated with the same concentrations of cisplatin, Prx II antisense enhanced cisplatin-induced cell death. The combination index (CI) at all doses of the combination was below 1, indicating that Prx II antisense sensitized cisplatin-induced cell death. This synergism was also observed in the cells transfected with a Prx II antisense oligomer. Our present results, therefore, suggest that Prx II antisense would be a very good sensitizer for cisplatin, and that Prx II as a target for chemosensitizers constitutes a promising avenue for future research.**

**Keywords:** Prx II, cisplatin, chemosensitizer, antisense, multidrug resistance

## Introduction

Prx II is an antioxidant enzyme that reduces H<sub>2</sub>O<sub>2</sub> and

other reactive oxygen species using thioredoxin as the immediate electron donor (Chae *et al.*, 1994), and its peroxidase activity prevents cells from reactive oxygen species insult (Chae and Rhee, 1999). Prx II is also involved in the cellular signaling pathways of growth factors and tumor necrosis factor- $\alpha$ , by virtue of its regulation of intracellular H<sub>2</sub>O<sub>2</sub> (Kang *et al.*, 1998; Sen, 1998). Six Prx isoforms have been identified which, based on the amino acid sequences, are generally divided into two subfamilies; groups I, II, III, and IV with two conserved cysteines and groups V and VI with one conserved cysteine (Butterfield *et al.*, 1999). Prx I and Prx II proteins are known to be located in cytoplasm (Chae *et al.*, 1999).

Prx overexpression is frequently observed in certain types of cancer tissues. Three types of Prx (I, II, and III) have been shown to be overexpressed in the case of human breast cancer, and it has been suggested that their overexpressions are related to cancer development or progression (Noh *et al.*, 2001). The increased expression of Prx I is also detected in lung cancer, thyroid tumors and oral cancer, and is suggested to constitute a potential tumor marker (Yanagawa *et al.*, 1999; Yanagawa *et al.*, 2000; Chang *et al.*, 2001). Prx II is upregulated by H<sub>2</sub>O<sub>2</sub> and cisplatin treatment, and its increased expression inhibits the apoptosis induced by cisplatin, thyrotropin, serum deprivation, ceramide, or etoposide, thereby rendering tumor cells resistant to some chemotherapeutic agents (Zhang *et al.*, 1997; Kim *et al.*, 2000; Chung *et al.*, 2001). We demonstrated earlier that Prx II was involved in radioresistance (Park *et al.*, 2000). In the present study, therefore, we explored the possibility as to whether inhibition of Prx II expression with antisense or drugs would accelerate cell death induced by anticancer agents.

## Materials and Methods

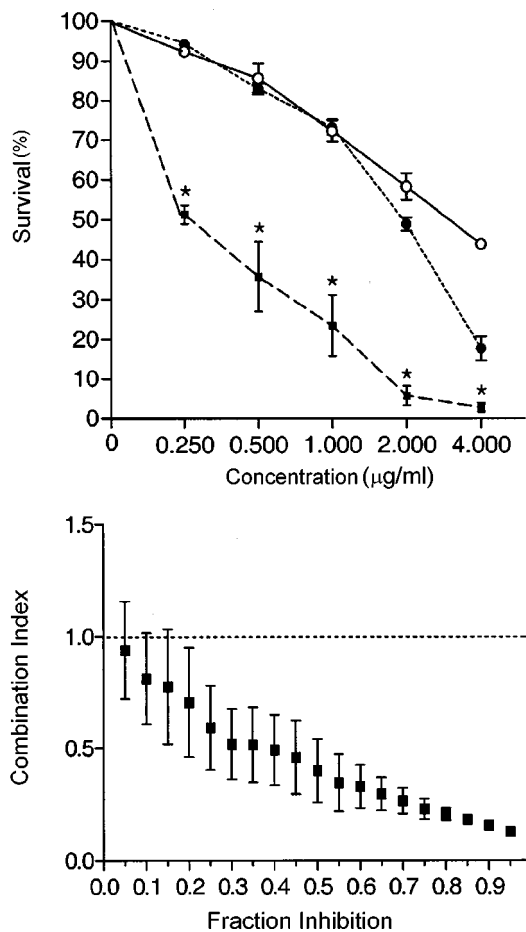
### Drug sensitivity assay

Human gastric-carcinoma cells (SNU638), were cultured at 37°C under 5% CO<sub>2</sub> in an RPMI 1640 medium (GIBCO/BRL, Grand Island, NY), containing 10% heat-inactivated fetal bovine serum, sodium bicarbonate (2 mg/ml), penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml). Exponentially growing SNU638 cells ( $2 \times 10^5$ ) were transferred to 60-mm culture dishes, and the cells were then transfected with various concentrations of the antisense Prx II expression vector (pPrxII/AS) or with antisense/sense oligomers combined with a mixture of cationic liposome (GIBCO/BRL). The cells were added

to the culture medium and then cultured for 16 h. The process of transfection was then repeated a second time. The cells were treated with various doses of cisplatin 16 h after transfection. After 4 days of incubation at 37°C, surviving cells were counted under a microscope.

### Determination of apoptotic cell death

SNU638 cells ( $1 \times 10^6$ ) were transferred to 100-mm culture dishes and the cells were transfected with 0.5  $\mu\text{g}/\text{ml}$  of the antisense Prx II expression vector (pPrxII/AS) along with a mixture of 5  $\mu\text{g}$  of cationic liposome (GIBCO/BRL), and the process was repeated a second time. After 16 h, the cells were treated with various doses of cisplatin, cultured for another 2 days, and then



**Figure 1.** Cisplatin sensitization of SNU638 cells after transfection with an antisense Prx II expression plasmid (pPrxII/AS). Cells were transfected with 0.25, 0.5, 1, 2, and 4  $\mu\text{g}/\text{ml}$  of pPrxII/AS respectively, as described in Materials and Methods. The cells were then treated with 0.25, 0.5, 1, 2, and 4  $\mu\text{g}/\text{ml}$  of cisplatin, respectively. After 4 days, cell survival was measured by cell counting and same experiments were repeated three times. Panel A: Survival of cells after treatment with pPrxII/AS (○) or cisplatin (●) or pPrxII/AS + cisplatin (■). Panel B: Fa-CI plot. A CI value < 1 represents synergy between Prx II antisense and cisplatin. Statistical significance between pPrxII/AS and pPrxII/AS+cisplatin, and between cisplatin and pPrxII/AS + cisplatin was analyzed. \*  $P < 0.01$ .

harvested. They were stained with propidium iodide and apoptotic cell death was then analyzed by flow cytometric analysis (FACscan; Becton Dickinson, Mountain View, CA), according to the manufacturer's instruction.

### Data analysis

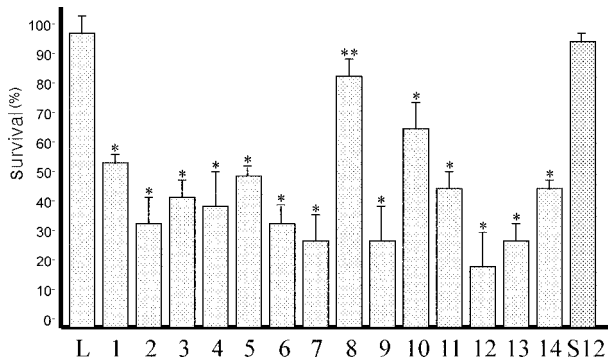
All results are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis between each group was performed by one-way analysis of variance (ANOVA). Statistical significance was accepted as  $P < 0.05$ . The combined effect of Prx II antisense and cisplatin was determined by dose-effect analysis, calculated using a microcomputer (Biosoft, Cambridge, England). A combination index (CI) value < 1 represents synergy between Prx II antisense and cisplatin, a CI value > 1 indicates antagonism, and a CI value of 1 means that the effects of the two agents are additive.

### Results

SNU638 cells were transfected with various concentrations of plasmid, pPrxII/AS, and then treated with the same concentrations of cisplatin. After incubation for 4 days, the cells were counted under a microscope. As shown in Figure 1A, pPrxII/AS itself caused cell death, which is consistent with our previous results on enhanced radiation-induced cell death by pPrxII/AS (Park *et al.*, 2000). Our previous results also demonstrated that pPrxII/AS inhibited Prx II expression (Park *et al.*, 2000; Chung *et al.*, 2001). pPrxII/AS and cisplatin caused 45.1% and 53.5% cell death, respectively, at a dose of 2  $\mu\text{g}$ , when used separately. However, combined treatment with these two agents caused 99.1% of cell death, suggesting

**Table 1.** Sequences of Prx II antisense oligomers

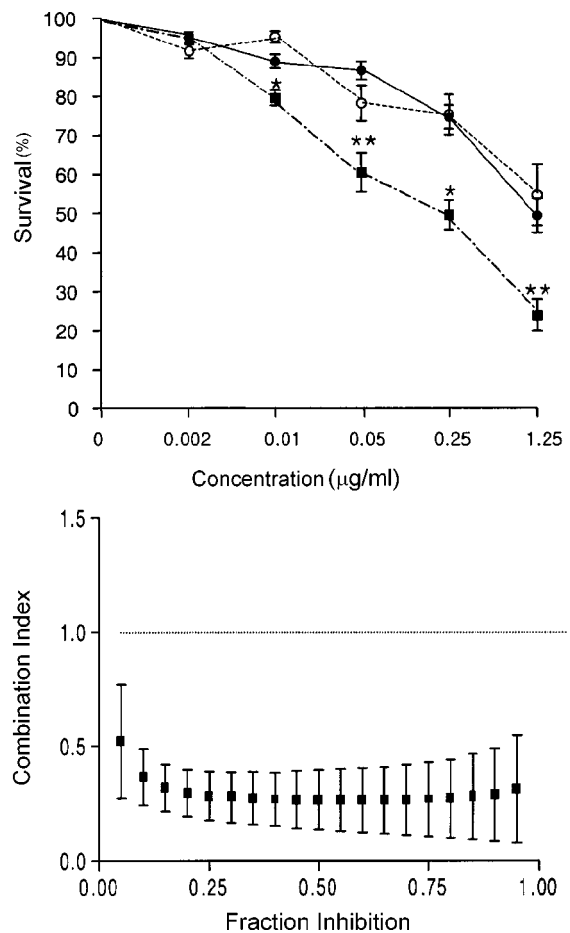
Name	Sequence
AS-1	CAGGTGGGGGCACAGGTGGA
AS-2	TCCGTTAGCCAGCCTAATTG
AS-3	GTTGGGCTTAATCGTGTACAC
AS-4	GTCTGTGTACTGGAAGGCCT
AS-5	CAAATCATTAAACAGTGATCT
AS-6	GTAGGCAATGCCCTCATCTG
AS-7	ACGCCGTAATCCTCAGACAA
AS-8	GTCACCAAGCAGGGGGATGT
AS-9	CCAAGCCAGGTGGTTGAACT
AS-10	GCGGAAGTCCTCTGCACGGT
AS-11	GTCCAGAGGGTAGAAAAAGA
AS-12	TTTGAAGGCCCATCAACCA
AS-13	CGCGCGTTACCGGAGGCCAT
AS-14	AGCTGCGTGGGCAAAGGCTA



**Figure 2.** Cell survival in Prx II antisense oligomers-treated SNU638. The cells were transfected twice with each 1 mM antisense oligomers and a sense oligomer as a control. After 4 days, survival curve was determined by cell counting and same experiments were repeated three times. ASs, cells transfected with the corresponding antisense oligomers; S12, cells transfected with S12; L, cells transfected with liposome only. Statistical significance between a sense oligomer (S12) and each antisense oligomers was analyzed. \* $P < 0.01$ , \*\* $P < 0.05$ .

that pPrxII/AS enhanced cisplatin-induced cell death. To confirm this synergistic effect, a CI plot was drawn. Figure 1B shows the CI value below 1 at all doses of the combination, indicating synergism.

In the above experiments, we used the full-length Prx II antisense. In order to examine whether antisense oligomers were also able to sensitize cisplatin-induced cell death, we prepared 14 antisense oligomers, which are listed in Table 1, and carried out similar experiments. Figure 2 shows that all 14 antisense oligomers induced cell death following transfection. Out of the 14 oligomers, we selected the AS12 oligomer as the best antisense oligomer for inducing cell death, and used it for sensitization experiments: One  $\mu\text{M}$  of AS12 induced ~80% cell death. Treatment of the cells with a Prx II sense oligomer (S12), which is complimentary to AS12, did not alter cell viability, thereby demonstrating that Prx II antisense itself was responsible for cell death. On the other hand, when SNU638 cells were transfected with AS12, the higher the dosage of AS12 in the cells, the greater the cell death followed (Figure 3A). When the cells were treated with these two agents together, 82.9% of cell death was observed, whereas AS12 or cisplatin alone, at a dose of 1.25  $\mu\text{g}/\text{ml}$ , caused 50.0% or 42.4% cell death, respectively, suggesting that the Prx II antisense oligomer also enhanced cisplatin-induced cell death. A CI plot indicated that at all doses of the combination, except for the case of 10% inhibition, the CI was below 1, thus confirming the synergistic effect (Figure 3B). To demonstrate the effect of Prx II antisense on apoptosis, the cells transfected with pPrxII/AS, following treatment with cisplatin, was examined by flow cytometry. Figure 4 shows that treatment of the transfected cells with 5  $\mu\text{g}/\text{ml}$  of cisplatin for 48 h induced 32.50% of apoptosis, whereas 26.13% of apoptosis was induced in the cells transfected

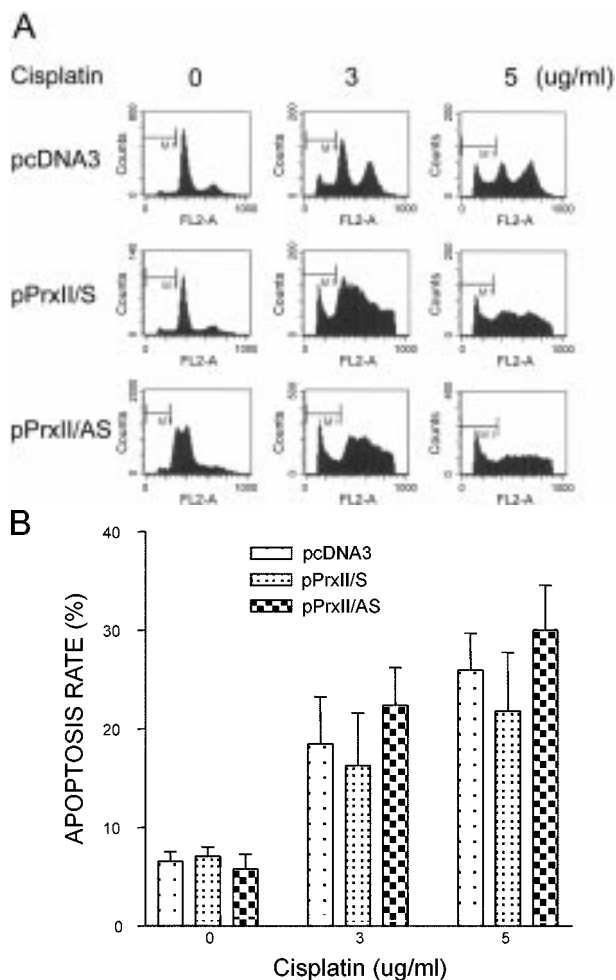


**Figure 3.** Synergistic effect of an antisense Prx II oligomer, AS-12, combined with cisplatin. Cells were transfected with 0.002, 0.01, 0.05, 0.25, and 1.25  $\mu\text{g}/\text{ml}$  of pPrxII/AS, respectively. The cells were then treated with 0.002, 0.01, 0.05, 0.25, and 1.25  $\mu\text{g}/\text{ml}$  of cisplatin, respectively. After 4 days, cell survival was measured by cell counting and same experiments were repeated three times. Panel A: Survival curve of cells after treatment with pPrxII/AS (○) or cisplatin (●) or pPrxII/AS + cisplatin (■). Panel B: Fa-CI plot. Statistical significance between pPrxII/AS and pPrxII/AS+cisplatin, and between cisplatin and pPrxII/AS+cisplatin was analyzed. \* $P < 0.01$ , \*\* $P < 0.05$ .

with pPrxII/S. These results suggest that Prx II antisense can be used as a chemosensitizer.

## Discussion

Prx II is an isotype of thioredoxin-dependent peroxidase, which reduces intracellular hydrogen peroxide that are detrimental to cells (Chae *et al.*, 1994). Prx II was known to be induced by exogenous  $\text{H}_2\text{O}_2$  generated by thyrotropin, serum deprivation, ceramide, or etoposide, possibly through eliminating intracellular  $\text{H}_2\text{O}_2$  and other reactive oxygen species (Kim *et al.*, 1997; Zhang *et al.*, 1997; Kim *et al.*, 2000), that are formed as a result of cisplatin treatment or radiation exposure (Sodhi and Gupta, 1986; Park *et al.*, 2000; Leach *et al.*, 2001). We



**Figure 4.** Apoptosis of cells treated with pPrxII/AS and cisplatin. Cells were transfected twice with 0.5 µg/ml of pPrxII/AS. After 16 h, the cells were treated with 3 and 5 µg/ml of cisplatin, respectively. After 2 days, the cells were harvested and stained with propidium iodide, and cell-cycle distribution was then analyzed by flow cytometric analysis. Panel A, cell-cycle distributions; panel B, apoptosis rates of cells after treatment with pPrxII/AS + cisplatin or pPrxII/S + cisplatin.

earlier showed that increased expression of Prx II was directly related to cisplatin resistance, and suggested that increased activity of Prx II might confer cancer cells with chemoresistance (Chung *et al.*, 2001) or radioresistance (Park *et al.*, 2000). This resistance seemed to be derived from the presence of an increased amount of Prx II, enabling the elimination of hydrogen peroxide produced as a consequence of cisplatin or radiation treatment. Cells resistant to H<sub>2</sub>O<sub>2</sub> were reported to be resistant to cisplatin (Spitz *et al.*, 1993), and down-regulation by Prx II antisense sensitized radiation-induced cell death (Park *et al.*, 2000). Indeed, as shown in the present results, Prx II antisense effectively enhanced cisplatin-induced cell death. Even though, in this report, we showed only that Prx II anti-sense sensitized cisplatin-induced cell death, we suggest that Prx II antisense could also enhance

other instances of anticancer drug-induced cell death, especially in the case of anticancer drugs which generate reactive oxy-gen species. This observation is consistent with previous results (Zhang *et al.*, 1997) where Prx II overexpression inhibited apoptosis in Molt-4 leukemia cells and functioned in a similar manner to Bcl-2. Also, Prx II overexpression rendered gastric cancer and ECV304 cells more resistant to chemotherapeutic drugs (Kang *et al.*, 1998).

Cellular resistance to anticancer drugs and  $\gamma$ -radiation is one of the difficulties encountered in the effective treatment of numerous cancers. Therefore, the enhancement of cellular sensitivity toward chemo- or radiation therapy constitutes an important field of research for improving cancer treatments. Because blocking the expression of Prx II disrupts total cellular redox homeostasis and induces apoptosis (Sato *et al.*, 1995), Prx II could be the focus of new drugs for use in the treatment of cancer. Our data, presented herein, strongly suggest that inactivation of the stress-activated protein Prx II constitutes a promising approach to the development of improved cancer treatments, and that inhibitors of Prx II represent very good anticancer drug candidates, especially in the role of chemosensitizers or radio-sensitizers.

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