*Mol. Cells*, Vol. 10, No. 1, pp. 38–46

Molecules and Cells



# Reactive Oxygen Species-specific Mechanisms of Drug Resistance in Paraquat-resistant Acute Myelogenous Leukemia Sublines

**Cheol-Hee Choi*, Hyang-Sook Kim, Oh-Seong Kweon, Tae-Bum Lee, Ho-Jin You, Han-Sik Rha, Jae-Hwan Jeong, Dong-Yoon Lim, Young-Don Min[1], Myung-Suk Kim[2], and Myung-Hee Chung[2]**

*Department of Pharmacology, Chosun University Medical School, Kwangju 501-759, Korea;*
*[1] Department of General Surgery, Chosun University Medical School, Kwangju 501-759, Korea;*
*[2] Department of Pharmacology, Seoul National University College of Medicine, Seoul 110-799, Korea.*

*(Received on September 7, 1999)*

**Reactive oxygen species (ROS)-specific mechanisms of drug resistance were explored in paraquat (PQ)-resistant acute myelogenous leukemia cell (OCI/AML-2) sublines. For this, PQ-resistant AML sublines, AML-2/PQ100 and AML-2/PQ400, were selected in the presence of PQ concentrations of 100 μg/ml and 400 μg/ml, respectively. They showed a moderate level of cross resistance to cisplatin and doxorubicin. They were also slightly more resistant than the parental cell (AML-2/WT) to etoposide, camptothecin and daunorubicin. The resistance of PQ-resistant AML-2 sublines to cisplatin seemed to be due to increased amounts of metallothionein, which was not only supported by reversal of resistance to cisplatin by propargylglycin (an inhibitor of metallothionein synthesis) but also confirmed by Western blot analysis and reverse transcription-PCR assay. In addition, both AML-PQ100 and /PQ400 sublines showed increased activities of Cu-, Zn-containing superoxide dismutase (Cu,Zn-SOD) and Mn-containing superoxide dismutase (Mn-SOD), whereas AML-2/PQ400, but not AML-2/PQ100, showed increased glutathione S-transferase activity as compared to that of AML-2/WT. However, there was no difference in other ROS-related cellular antioxidants between AML-2/WT and its PQ-resistant sublines. Taken together, these results strongly suggest that increases in levels of metallothionein, glutathione S-transferase, Cu,Zn-SOD and Mn-SOD play important roles in protective mechanisms against toxicity of PQ or ROS in AML cells.**

**Keywords:** Acute Myelogenous Leukemia; Glutathione S-Transferase; Metallothionein; Paraquat; Reactive Oxygen Species; Superoxide Dismutase.

* To whom correspondence should be addressed.
Tel: 82-62-220-3660; Fax: 82-62-232-4045
E-mail: chchoi@mail.chosun.ac.kr

## Introduction

Drug resistance has been one of the major causes of failure in clinical cancer chemotherapy. Several mechanisms, either alone or in combination, have been proposed to explain cellular drug resistance. These include overexpression of P-glycoprotein (Pgp) (Endicott and Ling, 1989; Riordan *et al*., 1985) and/or multidrug resistance-associated protein (MRP) (Cole *et al*., 1992), an increase in the reduced glutathione content (Hamilton *et al*., 1985), overexpression of glutathione S-transferase (Baist *et al*., 1986; Tew, 1994) or glutathione peroxidase (Krall *et al*., 1991), and alteration of topoisomerase II activity (Beck 1989; Isabella *et al*., 1991).

To overcome drug resistance, it is therefore very important to know which mechanisms or genes are involved in the resistance of certain types of cancers to each drug. Acute myelogenous leukemia (AML) has still shown a poor prognosis, at least in part, due to the development of drug resistance. Anthracycline antibiotics are clinically one of the important anticancer drugs in treating patients with AML. A variety of agents reduce $O_2$ univalently, producing reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals. which are potentially cytocidal

Abbreviations: AML, acute myelogenous leukemia; MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein; PQ, paraquat; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SOD, superoxide dismutase; WT, wild type.

(Fridovich, 1978). Anthracyclines have been also known to show cytotoxicity by generating ROS, although their poisoning of DNA topoisomerase II constitutes their major antitumor action (Lazo and Larner, 1998). Mechanisms by which anthracyclines exert multidrug-resistant phenotype are widely known to be the overexpression of drug efflux pumps such as Pgp and MRP. But once drug efflux pumps are overexpressed, they result in decreased intracellular concentrations of drugs, which make it difficult to study other mechanisms of drug resistance. This problem means that another drug is needed which can not only generate ROS but also possibly bypass overexpression of the drug efflux pumps favoring lipophilic compounds. One of these agents is methyl viologen (paraquat, PQ), a hydrophilic agent which promotes a flux of superoxide radicals within cells by transferring a single electron catalytically from biological reductants to $O_2$ both in procaryotes and in eukaryotes (Bagley *et al.*, 1986; Hassan and Fridovich, 1978; Kappus and Sies, 1981; Krall *et al*., 1988). We had selected resistant AML sublines for resistance to ROS by exposing PQ. Since PQ may exert its chemoresistant and radioresistant effects by induction of antioxidant enzymes (Jaworska *et al*., 1993), we examined the levels of cellular antioxidants which have been known to be related to ROS. The present study reveals that metallothionein, glutathione S-transferase, CuZn-SOD, and Mn-SOD play improtant roles in protective mechanisms against toxicity of PQ or ROS in AML cells.

## Materials and Methods

**Culture and selection for resistance to PQ** The OCI-AML-2 line obtained from the Ontario Cancer Institute (Toronto, Canada) was cultured at 37°C in a 5% $CO_2$ atmosphere using a-MEM medium (Gibco) with 10% heat inactivated fetal bovine serum (Sigma). Cells were maintained as a suspension culture, and subcultured. The PQ-resistant AML-2 sublines were selected from the parental wild-type AML-2 cell line (AML-2/WT) by chronic exposure to PQ (Sigma) in intermittent dosages schedule at sufficient intervals to permit the expression of resistance phenotypes. PQ was started from 1 × $IC_{50}$ (28 μg/ml), escalated at an increasing rate of 50%, and then cultured finally at the fixed concentrations (100 and 400 μg/ml) of PQ.

**Cytotoxicity assay** The *in vitro* cytotoxicity of drugs was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma] assay described by Pieters *et al*. (1988). The 50% inhibitory concentration ($IC_{50}$) for a particular agent was defined as the drug concentration which results in a 50% reduction in cell number as compared to the untreated control. $IC_{50}$ values were determined directly from semilogarithmic dose-response curves. Experiments were carried out at least twice.

**Treatment of propargylglycine** Propargylglycine (5 mM) had been treated a day before cisplatin was added in cells. After 3-day incubation with cisplatin, MTT assay was carried out to examine the effect of propargylglycine on the cross-resistance of PQ-resistant AML-2 sublines to cisplatin.

**Activities of antioxidant enzymes** Cellular lysates for assays of activity of catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase, glucose-6-phosphate dehydrogenase were prepared by washing with phosphate buffered saline (pH 7.4), then disrupting the cells in 50 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, 0.2% Triton-X100 and 1 mM phenylmethyl-sulfonyl fluoride by sonication. Unlysed cells and organelles were sedimented by centrifugation at 12,000 rpm for 15 min at 4°C using a microfuge.

The catalase was assayed spectrophotometrically by the catalase-induced loss of absorbance of 30 mM $H_2O_2$ at 240 nm at 25°C in 50 mM potassium phosphate buffer (pH 7) (Aebi, 1974). Glutathione peroxidase was assayed spectrophotometrically at 340 nm of 1 mM reduced glutathione to oxidized glutathione at the expense of 150 μM NADPH by glutathione reductase 0.24 U/ml in 100 mM potassium phosphate buffer (pH 7) at 37°C (Paglia & Valatine, 1967). Glucose-6-phosphate dehydrogenase was assayed specrophotometrically by measurement of the increase in absorbance at 340 nm as 200 μM NADP is reduced enzymatically at 25°C in 100 mM Tris·HCl (pH 8.0) with electrons derived from 0.83 mM glucose-6-phosphate (Kao and Hassan, 1985). Glutathione reductase was assayed by recording the loss of absorbance at 30°C at 340 nm as 100 μM NADPH is oxidized in the presence of 1.0 mM oxidized glutathione in 100 mM potassium phosphate buffer (pH 7.0) (Carlberg *et al*., 1975). Glutathione S-transferase was determined spectrophotometrically at 340 nm by measuring the formation of the conjugate in the presence of 1 mM glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 100 mM potassium phosphate buffer (pH 6.5) (Habig *et al*., 1974).

The spectrophotometrical analysis of SOD activities shows a wide range of variation, especially in the presence of cyanide added to distinguish Mn-SOD from Cu,Zn-SOD. The activity staining for superoxide dismutases was used in this study. Enzyme samples were separated on nondenaturing discontinuing polyacrylamide gels (4% stacking gel; 8% separating gel) and the SOD activity was visualized on these gels by the *in situ* staining technique described by Beauchamp and Fridovich (1971), which is based on the inhibition of the reduction of nitroblue tetrazolium by supeoxide radicals produced via photoreduction of riboflavin. Cu,Zn-SOD was distinguished from Mn-SOD simply by their different molecular weight or directly by treating the gels with KCN (2 mM) prior to photoreduction since the Cu,Zn-SOD is inhibited by cyanide which does not affect the Mn-SOD. Gels after SDS/PAGE were stained for SOD activity by incubation with gentle agitation in 2.45 mM nitroblue tetrazolium/28 mM N,N,N′,N′-tetra-methyl-ethylediamine/0.028 mM riboflavin/ 36 mM $K_2HPO_4$ at 37°C for 20 min in the dark, followed by illumination of a fluorescent light for about 5 min in the dark.

**Protein extraction and Western blot analysis** Total cell lysates were prepared by lysing harvested cells in extraction buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline) supplemented with 2 mM phenylmethylsulfonyl fluoride (Sigma) and 10 μg/ml leupeptin (Sigma). DNA was sheared by sonication. Sample buffer for

metallothionein analysis was used without 2-mercaptoethanol. Western blotting analysis was performed by a slight modification of the method first described by Towbin *et al.* (1979). Proteins were transferred onto a nitrocellulose membrane by electroblotting at a current of 60 V overnight. In the case of metallothionein, the transfer buffer containing 5% mercaptoethanol was used. The membrane was incubated in blocking solution (5% skim milk ) for 1 h at room temperature, washed, and then incubated with primary antibodies: mouse monoclonal antibody C219 (diluted 1:1000, Signet) for Pgp, rat monoclonal antibody Mrpr1 (1:1000, Signet) for MRP, mouse monoclonal antibody (1:500, Sigma) for metallothionein, sheep polyclonal antibody (1:1000, Biodesign) for Cu,Zn-SOD, sheep polyclonal antibody (1:200, Biodesign) for Mn-SOD and mouse monoclonal antibody (1:500, Oncogen) for Bcl-2. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibodies (diluted 1:1000) against each IgG of hosts of primary antibodies for 1 h. The membrane was then stained using the detection reagent of the ECL detection kit (Amersham, USA).

**RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) assay** All the RNA was extracted from the cells using the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1989). The expressions of *MDR1, MRP, GST, SOD1, SOD2, MT-II,* and β-*actin* genes was detected by the RT-PCR assay. Table 1 shows the nucleotide sequences for sense and antisense primers used in this study.

RNAs from each sample were reverse transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesta Research Laboratories) and oligo (dT) primer for 1 h at 37°C. The resulting cDNA was diluted 1:5 with water, then amplified with 2.5 units of *Taq* polymerase (Perkin-Elmer, USA) and 10 pmole of each primer in GeneAmp PCR2400 (Perkin-Elmer-Cetus) for 21 cycles (but 16 cycles for β-*actin*) of sequential denaturation (at 95°C for 30 s), annealing (at 65°C for *MDR1*, 53°C for *MRP* and β-*actin* , 55°C for *GST*, 50°C for *SOD1*, 61°C for *SOD2*, 57°C for *MT-II* ), and extension (at 72°C for 30 s). After the last cycle, all PCR products were subjected to a final extension for 5 min at 72°C. For quantitation, 5 μCi of [α-$^{32}$P] dCTP were added to each reaction mixture. After PCR, the products were combined and then electrophoresced on 7.5% nondenaturing polyacrylamide gels. The bands were scanned with a densitometer (Pdi, USA). The amounts of each mRNA transcript were normalized with that of β-*actin* mRNA.

**Protein determination** Protein concentration was determined using the Bradford protein assay with a Bio-Rad kit and standardized with bovine serum albumin.

**Statistical analysis** The statistical significance of the data was determined by t-test. P-values less than 0.05 were taken as statistically significant.

**Table 1.** Primer sequences used for PCR.

| Gene[1] | S&AS[2] | Nucleotide sequence | Sequence sregion[3] | Length of PCR products | References[4] |
|---|---|---|---|---|---|
| *MDR1* | S | 5′-CTGGTTTGATGTGCACGATGTTGG | 907–930 | 295 | Chen *et al*., 1986 |
| | AS | 5′-TGCCAAGACCTCTTCAGCTACTG | 1179–1201 | | |
| *MRP* | S | 5′-GACGGGAGCTGGGAAGTC | 4180–4197 | 389 | Cole *et al*., 1992 |
| | AS | 5′-ACAACCTACTCCGGTGCC | 4551–4568 | | |
| *GST -π* | S | 5′-CTCCGCTGCAAATACATCTC | 315–334 | 137 | Moscow *et al*., 1989 |
| | AS | 5′-ACAATGAAGGTCTTGCCTCC | 432–451 | | |
| *SOD1* | S | 5′-ATGGCGACGAAGGCCGTGTGC | 1–21 | 462 | Sherman *et al.*, 1983 |
| | AS | 5′-TTGGGCGATCCCAATTAC | 445-462 | | |
| *SOD2* | S | 5′-GGCATCAGCGGTAGCACCAG | 65–84 | 260 | Ho and Crapo, 1988 |
| | AS | 5′-TCTCCCTTGGCCAACGCCTC | 305–324 | | |
| *MT-II* | S | 5′-CCGGCTCCTGCAAATGCAAA | 47–66 | 220 | Lambert *et al*., 1996 |
| | AS | 5′-GTCACGGTCAGGGTTGTACA | 247–266 | | |
| β-*actin* | S | 5′-GACTATGACTTAGTTGCGTTA | 1912–1932 | 501 | Nakajima-lijima *et al*., 1985 |
| | AS | 5′-GTTGAACTCTCTACATACTTCCG | 2392–2412 | | |

[1] *MDR1*, Pgp gene; *GST*-p, glutathione S-transferase pi gene; *SOD1*, Cu,Zn-SOD gene; *SOD2*, Mn-SOD gene ; *MT-II*, metallothionein II gene.
[2] Sense and antisense.
[3] The oligonucleotide primers constructed for PCR correspond to the sense and antisense bases within these reported sequences.
[4] References for primer sequences.

## Results

**Selection of AML-2 sublines resistant to PQ and their cross-resistance to various anticancer drugs** PQ-resistant AML-2 sublines were selected by chronic exposure to gradually increasing concentrations of PQ from 28 μg/ml ($IC_{50}$ value) to 100 and 400 μg/ml intermittently. The PQ-resistant AML-2 sublines were designated AML-2/PQ100 and AML-2/PQ400. These two resistant sublines seemed to be similar to AML-2/WT in cell size. But they showed slightly longer doubling times of 25 h and 36 h, respectively, as compared to 22 h with AML-2/WT. This finding is consistent with the reports that intentionally developed resistant sublines have significantly longer cell doubling times than their parental cell lines (Choi and Ling, 1997; Nielsen *et al*., 1992). AML-2/PQ100, and /PQ400 were 3.6-fold and 14.5-fold more resistant to PQ, respectively, than AML-2/WT (Fig. 1 and Table 2). In addition, the PQ-resistant AML-2 sublines showed an intermediate level of resistance to cisplatin and doxorubicin but a low level of resistance to etoposide, camptothecin and daunorubicin. Paradoxically, both the PQ-resistant AML-2 sublines were more sensitive to vincristine than AML-2/WT (Table 2).

**Examination of the expression pattern of membrane transporters** The expression pattern of membrane transporters such as Pgp and MRP was determined by the RT-PCR assay. Neither AML-2/WT nor PQ-resistant AML-2 sublines expressed Pgp (data not shown). There was no difference in the amounts of MRP between AML-2/WT and PQ-resistant AML-2 sublines (data not shown).

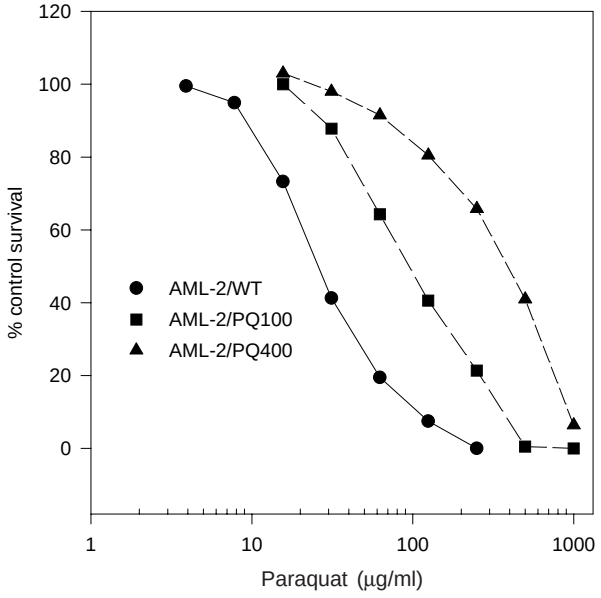


**Fig. 1.** Sensitivity of AML-2/WT and PQ-resistant AML-2 sublines to PQ. Cytotoxicity was determined by the MTT assay. WT, wild-type.

**Table 2.** Sensitivity of AML-2/WT and PQ-resistant AML-2 sublines to PQ and various anticancer drugs.

| Drug | AML-2/WT | AML-2/PQ100[a] | AML-2/PQ400[a] |
|---|---|---|---|
| | $IC_{50}$[b] | Relative resistance (fold)[c] | |
| PQ | 28.3 μg/ml | 3.6* | 14.5* |
| Doxorubicin | 7.6 ng/ml | 1.2 | 3.6* |
| Cisplatin | 229.0 ng/ml | 1.9* | 2.7* |
| Etoposide | 100.4 ng/ml | 1.6* | 1.8* |
| Camptothecin | 2.4 ng/ml | 1.5* | 1.8* |
| Daunorubicin | 15.6 ng/ml | 1.3* | 1.5* |
| Vincristine | 12.0 ng/ml | 0.6* | 0.4* |

[a] Numbers refer to selecting drug concentration in μg/ml.
[b] The drug concentration which results in a 50% reduction in cell number as compared to the untreated control.
[c] Ratio of $IC_{50}$ of PQ-resistant AML-2 subline to $IC_{50}$ of AML-2/WT.
* $P < 0.05$ compared with AML-2/WT cell line using the t-test.

**Cellular content of antioxidant enzymes and their expression** One of the proposed mechanisms for the cytotoxic effects of PQ suggests that the effect is mediated through the formation of intracellular superoxide radicals (Bagley *et al*., 1986; Krall *et al*., 1988). It is therefore possible that PQ resistance is associated with increased intracellular enzyme capacity to convert these superoxide radicals to inactive metabolites. The relative activities of antioxidant enzymes were determined in AML-2 /WT and PQ-resistant AML-2 sublines. Both the AML-2/PQ100 and /PQ400 sublines showed increased activities of Cu, Zn-SOD and Mn-SOD (Fig. 2), whereas AML-2/PQ400, but not AML-2/PQ100, increased glutathione S-transferase activity by 48% of that of AML-2/WT (Table 3).

The increased enzyme activities were due to the increased amount of the immunoreactive enzyme proteins as measured by Western blot analysis. Western blot analysis showed that the amounts of Cu,Zn-SOD and Mn-SOD in AML-2/PQ100 increased by about 1.7-fold and 3.4-fold, respectively, and in AML-2/PQ400 by 1.4-fold and 3.1-fold, respectively, as compared to those of AML-2/WT (Fig. 3). There were no differences in the steady-state levels of glutathione S-transferase, Cu,Zn-SOD and

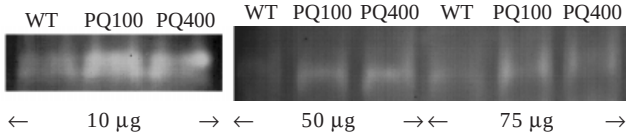


**Fig. 2.** Activity staining for Cu,Zn-SOD (**A**) and Mn-SOD (**B**) in AML-2/WT and PQ-resistant AML-2 sublines. Total cellular proteins were separated on a nondenaturing discontinuing polyacrylamide gel (4% stacking gel; 8% separating gel) and the SOD activity was visualized on the gel by the *in situ* staining technique described in Materials and Methods.

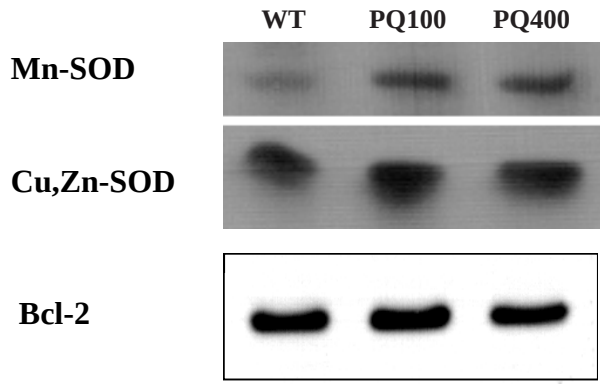


**Fig. 3.** Western blot analysis for Mn-SOD, Cu,Zn-SOD, and Bcl-2 in AML-2/WT and PQ-resistant AML-2 sublines. Ten μg and 75 μg of total cellular protein were separated respectively on 12% SDS/polyacrylamide gel for Western blot analyses of Cu,Zn-SOD and Mn-SOD. Fifty μg of protein was analyzed for Bcl-2 in a different blot.

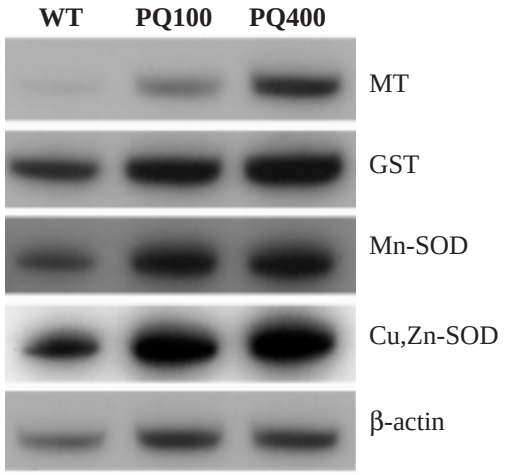


**Fig. 4.** Determination of mRNA transcripts in AML-2/WT and PQ-resistant AML-2 sublines by the RT-PCR assay. MT, metallothionein; GST, glutathione S-transferase.

Mn-SOD mRNAs as measured by the RT-PCR assay between AML-2/WT and AML-2/PQ100, while in AML-2/PQ400, they increased by 33%, 24% and 17%, respectively, as compared to those of AML-2/WT (Fig. 4). But there was no significant difference in the antioxidant enzymes including catalase, glutathione peroxidase, glucose-6-phosphate dehydrogenase between AML-2/WT and both PQ-resistant AML sublines (Table 3).

**Cellular content of metallothionein and the effect of propargylglycine on resistance to cisplatin in the PQ-resistant AML-2 sublines** Metallothionein was reported as possibly acting as a radical scavenger (Shiraishi *et al*., 1982; Thomas *et al*., 1986; Thornalley *et al*., 1985). The cellular content of metallothionein and the steady-state level of its mRNA were determined by Western blot and RT-PCR analyses, respectively. Western blot analysis showed that the cellular level of metallothionein in AML-2/PQ100 and /PQ400 increased by 25% and 70%, respectively, as compared to that of AML-2/WT (Fig. 5). The steady-state levels of metallothionein II mRNA also increased by 3.6-fold and 11.5-fold as compared to that of AML-2/WT (Fig. 4). In addition, the effect of propargylglycine, an inhibitor of metallothionein synthesis,

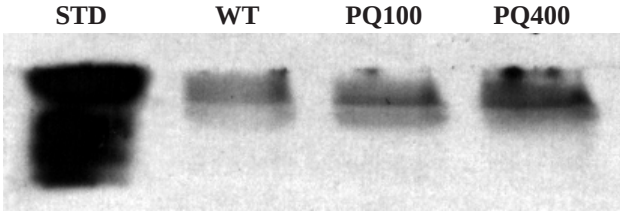


**Fig. 5.** Cellular content of metallothionein in AML-2/WT and PQ-resistant AML-2 sublines. Total cell extracts containing 100 μg protein were separated on a 15% SDS/polyacrylamide gel and then transferred on a nitrocellulose membrane in the presence of 5% mercaptoethanol. STD, authentic metallothionein standard (1 μg).

**Table 3.** Antioxidant enzyme activities in AML-2/WT and PQ-resistant AML-2 sublines.

| Enzyme | Mean specific activities[a] ± S.E.[b] (n = 3) | | |
|---|---|---|---|
| | AML-2 | AML-2/PQ100 | AML-2/PQ400 |
| Catalase | 38.1 ± 1.1 | 33.4 ± 2.9 | 45.8 ± 4.7 |
| Glutathione s-transferase | 113.1 ± 15.1 | 125.0 ± 8.2 | 168.2 ± 3.6* |
| Glutathione reductase | 42.9 ± 5.4 | 55.4 ± 13.6 | 56.4 ± 4.8 |
| Glutathione peroxidase | 99.4 ± 2.1 | 112.7 ± 11.5 | 119.1 ± 19.4 |
| Glucose-6-phosphate dehydrogenase | 56.3 ± 5.3 | 69.2 ± 3.2 | 56.3 ± 5.1 |

[a] expressed as nmol/min/mg protein except glutathione S-transferase (μmol/min/μg protein).
[b] standard error.
* $P < 0.02$ compared with AML-2/WT using the t-test.

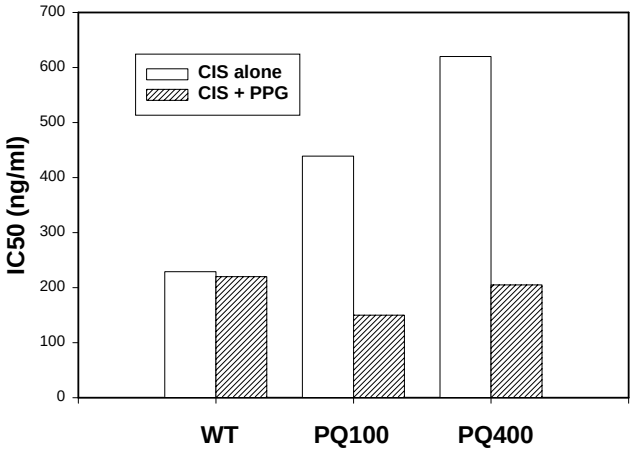


**Fig. 6.** Effect of propargylglycine on the resistance of AML-2/WT and PQ-resistant AML-2 sublines to cisplatin. $IC_{50}$ values were obtained from duplicate results of the MTT assay. PPG, propargylglycine; CIS, cisplatin

on the resistance of PQ-resistant AML-2 sublines to cisplatin was observed. Propargylglycine was able to completely reverse the cross-resistance of PQ-resistant AML-2 sublines to cisplatin (Fig. 6).

**Cellular content of Bcl-2** When Bcl-2 is overexpressed, cells are less sensitive to cytotoxic drugs. On the contrary, when it is underexpressed they are more sensitive (Desoize, 1994). To examine whether Bcl-2 would be involved in the resistance of PQ-resistant AML-2 sublines, the cellular content of Bcl-2 was determined by Western blot analysis. There was no difference in Bcl-2 levels between AML-2/WT and PQ-resistant AML-2 sublines (Fig. 3).

## Discussion

The ultimate aim of this study is to examine what ROS-specific mechanisms could be involved in the resistance of AML cells to anthracyclines. For this, we selected AML-2 sublines for resistance to PQ which can not only generate ROS but could also possibly bypass overexpression of the drug efflux pumps and then characterize PQ-resistant AML sublines. PQ-resistant AML-2 sublines also showed cross resistance to several cytotoxic drugs including cisplatin, doxorubicin, etoposide, camptothecin and daunorubicin by various degrees. From these findings, it could be suggested that common mechanisms would be involved in the toxicity and resistance for these drugs. By contrast, PQ-resistant AML-2 sublines were more sensitive to vincristine than was AML-2/WT. The specific changes in the PQ-resistant AML-2 sublines which sensitized these cells to vincristine remain to be determined. Paradoxically, increased sensitivity to anticancer drugs in the resistant cells has sometimes been reported. Doxorubicin-resistant P388 murine leukemic cells were significantly more sensitive to X-irradiation than were drug-sensitive P388 cells. Measured catalase activity in drug-resistant P388 cells was one-third of the activity measured in doxorubicin-sensitive P388 cells (Ramu *et al.*, 1984).

It has been proposed that PQ may exert its chemoresistant and radioresistant effects by induction of antioxidant enzymes (Jaworska *et al*., 1993). In the present study, increased cellular antioxidants such as metallothionein, superoxide dismutases, and glutathione S-transferase are involved in the resistance of PQ-resistant AML-2 sublines, but not glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase and Bcl-2. Chronic exposure ($>$ 200 d) of HA1 fibroblast to increasing concentrations of $H_2O_2$ or $O_2$ resulted in the development of a stable oxidative stress-resistant phenotype characterized by increased cellular antioxidants, including glutathione, glutathione reductase activity, glutathione peroxidase activity, SOD activity, and catalase activity (Spitz *et al*., 1990; 1992; Sullivan 1992). Recently, it has been suggested that many other anticancer drugs such as mitomycin C, nitrogen mustards, and inonizing radiation result in oxidative stress (Hahn *et al.*, 1990; Lewis *et al*., 1988). Doxorubicin-resistant P388 cells had 1.5 times glutathione peroxidase activity as measured in drug-sensitive P388 cells. But no significant differences in SOD activity between these cell lines were observed. The activities of the SOD, glutathione-S-transferase and glutathione reductase were 24%, 15% and 38%, respectively, higher in doxorubicin-resistant Friend leukemic cells than in their sensitive counterparts (Crescimanno *et al*., 1991). From the above results, it seems that ROS-specific mechanisms of drug resistance vary according to the types of cells and drugs used.

In the present study, the cross-resistance of PQ-resistant AML-2 sublines to cisplatin suggests the possible involvement of metallothionein in multidrug resistance phenotype of both resistant AML-2 sublines, which was not only supported by the reversal effect of propargylglycine (a specific inhibitor of cystathionase) on cisplatin resistance but also confirmed by data from Western blot and RT-PCR analyses. Overexpression of metallothionein by PQ indicates that metallothionein may be induced by superoxide radicals or their byproducts. Cell lines expressing high metallothionein levels have been shown to be resistant to various anticancer drugs such as cisplatin, L-PAM, chlorambucil, adriamycin, and ionizing radiation (Basu *et al*., 1990; Nagamura *et al*., 1988). Recently, mouse embryonic fibroblasts lacking functional metallothionein showed enhanced sensitivity to anticancer drugs such as cisplatin, melphalan, bleomycin, cytarabine as compared to wild-type cells (Kondo *et al*., 1995). In addition, it was reported that transgenic mice expressing

metallothionein activity 10- or 130-fold higher than nontransgenic control exhibited a significant resistance to *in vivo* doxorubicin-induced cardiotoxicity (Kang *et al.*, 1997). Propargylglycine could inhibit metallothionein synthesis in a mouse bladder tumor model and decrease cisplatin resistance acquired by an increase in the metallothionein level of the tumor, suggesting that metallothionein plays a role in the drug resistance of certain tumors (Satoh *et al.*, 1993; 1994).

In the present study, both AML-2/PQ100 and/PQ400 sublines showed increased activities of both Cu,Zn-SOD and Mn-SOD, which was consistent with a previous report that PQ-resistant HeLa cells PQRHM40 were isolated for resistance to 40 μM resisted to toxicity of PQ by increasing the cellular activity of Cu,Zn-SOD and Mn-SOD (Krall *et al*., 1988).

Meanwhile, AML-2/PQ400, but not AML-2/PQ100, showed increased activity of glutathione S-transferase as compared to that of AML-2/WT. This suggests that cells need more glutathione S-transferase to resist the toxicity of the high concentration of PQ. The glutathione S-transferase can catalyze the conjugation of electrophilic substances with the ubiquitous nucleophile GSH and the non-selenium-dependent reduction of organic peroxides (Hayes, 1995; Tew, 1994). Although it is not known definitively that GSH conjugates can be formed from PQ, it is proposed that PQ could form ROS and subsequent lipid peroxides which could be conjugated with glutathione by glutahione S-transferase. There have been numerous reports showing that the expression of glutathione S-transferase is elevated in various cultured cell lines tissues possessing resistance to anticancer drugs such as adriamycin, melphalan, cisplatin, cyclophosphamide, and chlorambucil, as well as *in vivo* cancers that have become resistant to therapy after administration of anticancer drugs (Tew, 1994). The transfection of antisense cDNA of glutathione S-transferase into adriamycin-resistant colon cancer cells resulted in the reduction of intracellular glutathione S-transferase concentration (by about half) and an increased sensitivity to adriamycin (4.4-fold) (Ban *et al*., 1996).

The present data about catalase are in agreement with previous studies, in which catalase has not usually been connected to drug resistance (Coursin *et al*., 1996; Sinha and Mimnaugh, 1990). It has been known that Bcl-2 possesses activities to stimulate antioxidant protection and to delay processes leading to cell death in mammalian cells (Hockenbery *et al*., 1993; Longo *et al*., 1997). But Bcl-2 was not involved in the resistance of PQ-resistant AML-2 sublines.

In conclusion, the present study suggests that the increased levels of metallothionein, glutathione S-transferase, Cu,Zn-SOD, and Mn-SOD are involved in protective mechanisms against toxicity of PQ or ROS in AML cells.

**Acknowledgments** We wish to thank Dr. S.-W. Jung, J.-C. You, Y.-K. Jung for critically reading the manuscript. This study was supported by the academic research fund of the Ministry of Education of Korea in 1997.

## References

Aebi, H. (1974) Catalase; in *Methods of Enzymatic Analysis*, Bermeyer, H. U. (ed.), Vol. 2, pp. 673–684, New York Academic Press Inc.

Bagley, A. C., Krall, J., and Lynch, R. E. (1986) Superoxide mediates the cytotoxicity of paraquat for Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* **83**, 3189–3193.

Baist, G., Tulpule, A., Sinha, B. K., Katki, A. G., Myers, C. E., and Cowan, K. H. (1986) Overexpression of a novel anionic glutathione transferase in multidrug resistant human breast cancer cells. *J. Biol. Chem.* **261**, 15544–15549.

Ban, N., Takahashi, Y., Takayama, T., Kura, T., Katahira, T., Sakamaki, S., and Niitsu, Y. (1996) Transfection of glutathione S-transferase (GST)-p antisense complementary DNA increases the sensitivity of a colon cancer cell line to adriamycin, cisplatin, mephalan, and etoposide. *Cancer Res.* **56**, 3577–3582.

Basu, A. and Lazo, J. S. (1990) A hypothesis regarding the protective role of metallothioneins against the toxicity of DNA interactive anticancer drugs. *Toxicol. Lett.* **50**, 123–135.

Beauchamp, C. and Fridovich, I. (1971) Superoxide dismutase. Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**, 276–287.

Beck, W. T. (1989) Unknotting the complexities of multidrug resistance; The involvement of DNA topoisomerases in drug action and resistance. *J. Natl. Cancer Inst.* **81**, 1683–1685.

Carlberg, I. and Mannervik, B. (1975) Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* **250**, 5475–5480.

Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson I. B. (1986) Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**, 381–389.

Choi, C. H. and Ling V. (1997) Isolation and characterization of daunorubicin-resistant AML-2 sublines. *Mol. Cells* **7**, 170–177.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V., and Deeley, R. G. (1992) Overexpression of a transporter gene in a multidrug resistant human lung cancer cell line. *Science* **258**, 1650–1654.

Coursin, D. B., Cihla, H. P., Oberley, T. D., and Oberley, L. W. (1996) An immunohistochemical analysis of antioxidant and glutathione S-transferase enzyme levels in normal and neoplatic human lung. *Histo. Histopathol.* **11**, 851–860.

Crescimanno, M., S'Alessandro, N., Armata, M. G., Toulmond, S., and Tapiero, H. (1991) Modulation of the antioxidant activities in dox-sensitive and -resistant Friend leukemia cells. Effect of doxorubicin. *Anticancer Res.* **11**, 901–903.

Desoize, B. (1994) Anticancer drug resistance and inhibition of apoptosis. *Anticancer Res.* **14**, 2291–2294.

Endicott, J. A. and Ling, V. (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.* **58**, 137–171.

Fridovich, I. (1978) The biology of oxygen radicals. *Science* **201**, 875–880.

Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974) Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130–7139.

Hamilton, T. C., Winker, M. A., Louie, K. G., Batist, G., Behrens, B. C., Tsuruo, T., Grotzinger, K. R., McKoy, W. M., Young, R. C., and Ozols, R. F. (1985) Augmentation of adriamycin, melphalan and cisplatin cytotoxicity in drug resistant and sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem. Pharmacol.* **34**, 2583–2584.

Hahn, P., Nevaldine, B., and Morgan, W. F. (1990) X-ray induction of methotrexate resistance due to *dhfr* gene amplification. *Somat. Cell Mol. Genet.* **16**, 413–423.

Hassan, H. M. and Fridovich, I. (1978) Superoxide radical and the oxygen enhancement of the toxicity of paraquat in *Escherichia coli*. *J. Biol. Chem.* **253**, 8143–8148.

Hayes, J. D. and Pulford, D. J. (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isozymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* **30**, 445–600.

Ho, Y.-S. and Crapo, J. D. (1988) Isolation and characterization of complementary DNAs encoding human manganese-containing superoxide dismutase. *FEBS lett.* **229**, 256–160.

Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241–152.

Isabella, P. D., Capranico, G., and Zunino, F. (1991) The role of topoisomerase II in drug resistance. *Life Sci.* **48**, 2195–2205.

Jaworska, A., Stojcevic-Lemic, N., and Nias, A. H. (1993) The effect of paraquat on the radiosensitivity of melanoma cells; the role of superoxide dismutase and catalase. *Free Radic. Res. Commun.* **18**, 139–145.

Kang, Y. J., Chen, Y., Yu, A., Voss-McCowan, M., and Epstein, P. N. (1997) Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. *J. Clin. Invest.* **100**, 1501–1506.

Kao, S. M. and Hassan, H. M. (1985) Biochemical characterization of paraquat tolerant mutant of *Escherichia coli*. *J. Biol. Chem.* **260**, 10478–10481.

Kappus, H. and Sies, H. (1981) Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. *Experientia.* **37**, 1233–1241.

Kondo, Y., Woo, E. S., Michalska, A. E., Choo, K. H. A., and Lazo, J. S. (1995) Metallothionein null cells have increased sensitivity to anticancer drugs. *Cancer Res.* **55**, 2021–2023.

Krall, J., Bagley, A. C., Mullenbach, G. T., Hallewell, R. A., and Lynch, R. E. (1988) Superoxide mediates the toxicity of paraquat for cultured mammalian cells. *J. Biol. Chem.* **263**, 1910–1914.

Krall, J., Speranza, M. J., and Lynch, R. E. (1991) Paraquat-resistant HeLa cells: Increased cellular content of glutathione peoxidase. *Arch. Biochem. Biophys.* **2867**, 311–315.

Lambert, E., Kille, P., Kay, J., and Swaminathan, R. (1996) Cloning and sequencing a novel metallothionein 1 isoform expressed in human reticulocytes. *FEBS Lett.* **389,** 210–212.

Lazo, J. S. and Larner, J. M. (1998) Individual antineoplatic drugs; in *Human Pharmacology, Molecular to Clinical*, 3rd ed., Brody, T. M., Larner, J., and Minneman, K. P. (eds.), p. 606, Mosby, New York.

Lewis, A. D., Hickson, I. D., Robson, C. N., Harris, A. L., Hayes, J. D., Griffiths, S. A., Manson, M. M., Hall, A. E., and Wolf, C. R. (1988) Amplification and increased expression of a class glutathione S-transferase-encoding genes associated with resistance to nitrogen mustards. *Proc. Natl. Acad. Sci. USA* **85**, 8511–8515.

Longo, V. D., Ellerby, L. M., Bredesen, D. E., Valentine, J. S., and Gralla, E. B. (1997) Human Bcl-2 reverses survival defects in yeast lacking superoxide dismutase and delays death of wild-type yeast. *J. Cell Biol.* **137**, 1581–1588.

Moscow, J. A., Fairchild, C. R., Madden, M. J., Ransom, D. T., Wieand, H. S., O'Brien, E. E., Poplack, D. G., Cossman, J., Myers, C. E., and Cowan, K. H. (1989) Expression of anionic glutathione-S-transferase and P-glycoprotein genes in human tissues and tumors. *Cancer Res.* **49**, 1422–1428.

Nagamura, A., Satoh, M., and Imura, N. (1988) Specific reduction of toxic side effects of adriamycin by induction of metallothionein in mice. *Jpn. J. Cancer Res.* **79**, 406–411.

Nakajima-lijima, S., Hamada, H., Reddy, P., and Kakunaga, T. (1985) Molecular structure of the human cytoplasmic *beta-actin* gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA* **82**, 6133–6137.

Nielsen, D. and Skovsgaard, T. (1992) P-glycoprotein as multidrug transporter: a critical review of current multidrug resistant cell lines. *Biochem. Biophys. Acta.* **1139**, 169–183.

Paglia, D. E. and Valatine, W. N. (1967) Studies on the quantitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70**, 158–169.

Pieters, R., Huismans, D. R., Leyva, A., and Veerman, A. J. P. (1988) Adaptation of the rapid automatic tetrazolium dye based (MTT) assay for chemosensitivity testing in childhood leukemia. *Cancer Lett* **41**, 323–332.

Ramu, A., Cohen, L., and Glaubiger, D. (1984) Oxygen radical detoxification enzymes in doxorubicin-sensitive and -resistant P388 murine leukemic cells. *Cancer Res.* **44**, 1976–1989.

Riordan, J. R., Deuchars, K., Kartner, N., Alon, N., Trent, J., and Ling, V. (1985) Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature* **316**, 817–819.

Satoh, M., Kloth, D. M., Kadhim, S. A., Chin, J. L., Naganuma, A., Imura, N., and Cherian, M. G. (1993) Modulation of both cisplatin nephrotoxicity and drug resistance in murine bladder tumor by controlling metallothionein synthesis. *Cancer Res.* **53**, 1829–1832.

Satoh, M., Cherian, G. C., Imura, N., and Shimizu, H. (1994) Modulation of resistance to anticancer drugs by inhibition of metallothionein synthesis. *Cancer Res.* **54**, 5255–5257.

Sherman, L., Dafni, N., Lieman-Hurwitz, J., and Groner, Y. (1983) Nucleotide sequence and expression of human chrmosome 21-encoded superoxide dismutase mRNA. *Proc. Natl. Acad. Sci. USA* **80**, 5465–5469.

Shiraishi, N., Utsumi, K., Morimoto, S., Joja, I., Iida, S., Takeda, Y., and Aono, K. (1982) Inhibition of nitroblue tetrazolium reduction by metallothionein. *Physiol. Chem. Phys.* **14**, 533–537.

Sinha, B. K. and Mimnaugh, E. G. (1990) Free radicals and anticancer drug resistance: oxygen free radicals in the mechanisms of drug toxicity and resistance by certain tumors. *Free Radical Biol. Med.* **8**, 567–581.

Spitz, D. R., Elwell, J. H., Sun, Y., Oberley, L. W., Oberley, T. D., Sullivan, S. J., and Roberts, R. J. (1990) Oxygen toxicity in control and $H_2O_2$-resistant Chinese hamster fibloblast cell lines. *Arch. Biochem. Biophys.* **279**, 249–260.

Spitz, D. R., Adams, D. T., Sherman, C. M., and Roberts, R. J. (1992) Mechanisms of cellular resistance to hydrogen peroxide and 4-hydroxy-2-nonrenal toxicity: the significance of increased catalase activity in $H_2O_2$-resistant fibloblasts. *Arch. Biochem. Biophys.* **292**, 221–227.

Sullivan, S. J., Oberley, T. D., Roberts, R. J., and Spitz, D. R. (1992) A stable $O_2$-resistant cell line: role of lipid peroxidation by-products in $O_2$-mediated injury. *Am. J. Physiol.* (*Lung Cell. Mol. Physiol.*) **262**, L748–L756.

Tew, K. D. (1994) Glutathione associated enzymes in anticancer

drug resistance. *Cancer Res.* **54**, 4313–4320.
Thomas, J. P., Bachowski, G. J., and Girotti, A. W. (1986) Inhibition of cell membrane lipid peroxidation by cadmium- and zinc-metallothioneins. *Biochim. Biophy. Acta*. **884**, 448–461.
Thornalley, P. J. and Vasak, M. (1985) Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydrogen redicals. *Biochim. Biophys. Acta.* **827**, 36–44.
Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellose sheets: procedures and some applications. *Pro. Natl. Acad. Sci. USA* **76**, 4350–4354.