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Biochemical Pharmacology 68 (2004) 1857–1867
Biochemical Pharmacology
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# Effect of change in cellular GSH levels on mitochondrial damage and cell viability loss due to mitomycin c in small cell lung cancer cells

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Received 4 February 2004; accepted 14 June 2004

**Abstract**

The effect of GSH depletion on mitochondrial damage and cell death due to mitomycin c (MMC) was assessed in small cell lung cancer (SCLC) cells. Cytotoxicity of MMC was attenuated by Tempol and dicumarol, inhibitors of the enzymatic reduction, and increased by xanthine oxidase. The MMC-induced cell death and decrease in the GSH contents in SCLC cells were inhibited by caspase inhibitors (z-DQMD.fmk, z-IETD.fmk and z-LEHD.fmk) and antioxidants (*N*-acetylcysteine, dithiothreitol and *N*-(2-mercaptopropionyl)glycine, melatonin, rutin and carboxy-PTIO). Thiol compounds, melatonin and rutin attenuated the MMC-induced nuclear damage, decrease in mitochondrial transmembrane potential, release of cytochrome *c* and activation of caspase-3. Treatment of MMC caused a significant decrease in GSH contents in SCLC cells, which was followed by increase in the formation of reactive oxygen species. Depletion of GSH due to L-buthionine sulfoximine enhanced the MMC-induced activation of caspase-3 and cell death in SCLC cells. Antioxidants, including *N*-acetylcysteine, depressed formations of nitric oxide, malondialdehyde and carbonyls due to MMC in SCLC cells. The results show that the reductive activation of MMC may cause cell death in SCLC cells by inducing mitochondrial dysfunction, leading to caspase-3 activation, and by activation of caspase-8. The MMC-induced change in the mitochondrial membrane permeability, followed by cell death, in SCLC cells may be significantly enhanced by decrease in the intracellular GSH contents due to oxidative attack of free radicals.


*Keywords:* Mitomycin c; Small cell lung cancer cells; Mitochondrial membrane permeability; Cell death; GSH depletion; Free radicals

## 1. Introduction

MMC is commonly used in combination with other drugs for the treatment of breast, lung and prostate cancer. This drug is activated by the enzymatic reduction of its quinone moiety and then binds covalently to DNA, which shows a cytotoxic effect on tumor or normal cells [1,2]. The enzymatic bioactivation produces ROS, such as superoxide radicals and hydrogen peroxide, which are involved in oxidative damage of cell components [3,4].

In addition to DNA, mitochondria are postulated as a cellular target for MMC. The membrane permeability transition of mitochondria has been shown to be involved in a variety of toxic and oxidative forms of cell injury as well as apoptosis. Opening of the mitochondrial permeability transition pore causes a depolarization of the transmembrane potential, release of $Ca^{2+}$ and cytochrome *c* and loss of oxidative phosphorylation, which results in loss of cell viability [5–7]. In EMT6 mouse mammary carcinoma cells, MMC damages the DNA and membrane integrity in mitochondria [8]. On the other hand, in human pulmonary adenocarcinoma A549 cells, the morphological changes of mitochondria due to MMC have not been demonstrated [9]. It is also uncertain that MMC-induced cell death is mediated by caspase-3 activation [10,11]. Therefore, it is necessary to clarify whether opening of the mitochondrial membrane permeability pore in cells exposed to MMC induces the activation of caspases through the release of cytochrome *c*.

*Abbreviations:* MMC, mitomycin c; SCLC cells, small cell lung cancer cells; MPG, *N*-(2-mercaptopropionyl)glycine; Carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; z-DQMD.fmk, z-Asp-(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone; z-IETD.fmk, z-Ile-Glu-(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone; z-LEHD.fmk, z-Leu-Glu-(O-ME)-His-Asp(O-Me) fluoromethyl ketone; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; $DCFH_2$-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescin; PMSF, phenylmethylsulfonylfluoride; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); MTT, 1,3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide; ROS, reactive oxygen species

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doi:10.1016/j.bcp.2004.06.010

Drops in GSH levels increase the sensitivity of cells to the toxic effect of toxic substances [12], and are associated with mitochondrial dysfunction [13]. The toxicity of anticancer drugs may largely depend on the intracellular level of reduced GSH. Depletion of GSH due to L-buthionine-(*S*,*R*)-sulfoximine increases the toxicity of etoposide on K562 human erythroleukemia cells [14]. In contrast to this report, the GSH depletion does not affect the toxicity of etoposide and doxorubicin on U-937 human promonocytic leukemia cells [15]. The mitochondrial membrane permeability is postulated to be affected by the redox state of dithiols [16]. Therefore, it is necessary to explore whether the MMC-induced change in the mitochondrial membrane permeability and cell death is modulated by GSH depletion.

Although mitochondria are suggested as a cellular target for MMC, the effect of GSH depletion against mitochondrial damage due to MMC has not been clarified. The purpose of the present study was to explore the influence of the GSH depletion against cytotoxicity of MMC in relation to the mitochondrial membrane permeability transition. We examined the toxic effect of MMC on SCLC cells by measuring the effect on the GSH contents, ROS formation, mitochondrial transmembrane potential, cytochrome *c* release and caspase-3 activity.

## 2. Materials and methods

### 2.1. Materials

MMC, *N*-acetylcysteine, MPG, melatonin, rutin trihydrate, carboxy-PTIO, z-DQMD.fmk, z-IETD.fmk, z-LEHD.fmk, Hoechst 33258, Tempol, $DiOC_6(3)$, $DCFH_2$-DA, PMSF, DTNB, glutathione reductase (from spinach), MTT, glutathione (reduced form, GSH) and RPMI were purchased from Sigma–Aldrich Inc. TiterTACS™ colorimetric apoptosis detection kit was purchased from Trevigen Inc., Quantikine® M human cytochrome *c* assay kit was purchased from R&D systems and ApoAlert™ CPP32/Caspase-3 assay kit from CLONTECH Laboratories Inc. The polystyrene 24- and 96-well plates (Corning®) were purchased from Corning Incorporated, and 15 ml polypropylene tubes (Falcon®) used in the isolation of cells were from Becton Dickinson.

### 2.2. Culture of small cell lung cancer cells

The human SCLC cells (NCI-H889) were obtained from the Korean cell line bank. Cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% $CO_2$ atmosphere at 37 °C. The culture medium was changed every 3 days, and the cells were subcultured about once a week. Cells ($1 \times 10^7$) were plated on polystyrene 60 mm × 15 mm cell culture dishes (Corning Incorporated), 48–72 h before experiments. Cells were washed with RPMI containing 1% FBS and re-plated onto 96-well plates at a density of $4 \times 10^4$ cells per well in a volume of 200 μl (or various numbers of cells/ml in 24-well plates). Cells were treated with MMC in RPMI containing 1% FBS for 24 h at 37 °C.

### 2.3. Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [17]. SCLC cells ($4 \times 10^4$) were treated with MMC for 24 h at 37 °C. The medium (200 μl) was incubated with 10 μl of 10 mg/ml MTT solution for 2 h at 37 °C. Culture medium was removed, and 100 μl of dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Spectra MAX 340). Cell viability was expressed as a percentage of the value in control cultures.

### 2.4. Morphological observation of nuclear change

SCLC cells ($1 \times 10^6$ cells/ml) were treated with MMC for 24 h at 37 °C, and the nuclear morphological change was assessed using Hoechst 33258 dye [18]. After treatment, the medium was centrifuged at 412 × *g* for 10 min in a microplate centrifuge, and the medium was removed. The pellets were washed twice with phosphate buffered saline (PBS). SCLC cells were incubated with 1 μg/ml Hoechst 33258 for 3 min at room temperature, and the nuclei were visualized using an Olympus Microscope with a WU excitation filter.

### 2.5. Measurement of apoptosis in cells

Apoptosis was assessed by measuring the DNA fragmentation, which occurs following the activation of endonucleases. SCLC cells ($1 \times 10^5$ cells/ml) were treated with MMC for 24 h at 37 °C, washed with PBS and fixed with formaldehyde solution. Nucleotide (dNTP) was incorporated at the 3′ ends of DNA fragments using terminal deoxynucleotidyl transferase (TdT). This nucleotide was detected using a horseradish-peroxidase and TACS-Sapphire according to TiterTACS protocol. Data were expressed as absorbance at 450 nm.

### 2.6. Measurement of total glutathione

The total glutathione (GSH + GSSG) was determined using glutathione reductase [19]. SCLC cells ($4 \times 10^4$ cells/ml) were treated with MMC for 24 h at 37 °C, centrifuged at 412 × *g* for 10 min in a microplate centrifuge, medium was removed, and the pellets were washed twice with PBS. Cells were dissolved with 2% 5-sulfosa-

licylic acid (100 μl) and then incubated in 100 μl of the reaction mixture containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM DTNB and 105 mM $NaH_2PO_4$, pH 7.5 at 37 °C. Glutathione reductase (20 μl, 100 U/ml) was added and the mixture incubated for a further 10 min. Absorbance was measured at 412 nm using a microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSH that was incubated in the mixture, as in samples.

### 2.7. Flow cytometric measurement of mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential during the MMC-induced apoptosis in SCLC cells were quantified by flow cytometry with the cationic lipophilic dye $DiOC_6(3)$ [20]. Cells ($1 \times 10^6$ cells/ml) were treated with MMC for 4 h at 37 °C. $DiOC_6(3)$ (40 nM) was added to the medium, and incubation was performed for 15 min at 37 °C. After centrifugation at 412 × *g* for 10 min, the supernatants were removed, and the pellets were re-suspended in PBS containing 0.5 mM EDTA. For analysis, a FACScan cytofluorometer (Becton Dickinson) with argon laser excitation at 501 nm was used to assess 10,000 cells from each sample.

### 2.8. Measurement of cytochrome c release

The release of cytochrome *c* from mitochondria into the cytosol was assessed by using a solid phase ELISA kit for the detection of human cytochrome *c*. SCLC cells ($5 \times 10^5$ cells/ml) harvested by centrifugation at 412 × *g* for 10 min were washed twice with PBS and re-suspended in 250 mM sucrose, 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol and 0.1 mM PMSF. Cells were further homogenized by successive passages through a 26-gauge needle. The homogenates were centrifuged at 100,000 × *g* for 30 min, and the supernatant obtained was used for the analysis of cytochrome *c*. The supernatants were added into the 96-well microplates coated with monoclonal antibody specific for human cytochrome *c* that contains cytochrome *c* conjugate. The procedure was performed as described in the assay kit. Absorbance of samples was measured at 450 nm in a microplate reader. A standard curve was constructed by adding the diluted solutions of cytochrome *c* standard, handled like samples, to the microplates coated with monoclonal antibody. The amount was expressed as ng/ml by reference to the standard curve.

### 2.9. Measurement of caspase-3 activity

The activation of caspase-3 occurred while the apoptotic process in cells was assessed [6]. SCLC cells ($2 \times 10^6$ cells/ml) were treated with MMC for 24 h at 37 °C, and caspase-3 activity was determined as described in user's manual of ApoAlert™ CPP32/Caspase-3 assay kit. The supernatant obtained by a centrifugation of cells dissolved was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and was incubated for 1 h at 37 °C. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. The standard curves were obtained from absorbances in the *p*-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as 1 nmol of chromophore *p*-nitroanilide produced.

### 2.10. Measurement of intracellular ROS formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent DCF by hydroperoxides, was used to measure relative levels of cellular peroxides [21]. After exposure to MMC, SCLC cells ($4 \times 10^4$) were incubated with 50 μM dye for 30 min at 37 °C, and then were washed with PBS. The cell suspensions were centrifuged at 412 × *g* for 10 min and medium was removed. Cells were dissolved with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN).

### 2.11. Measurement of nitrite/nitrate production

The formation of nitric oxide in SCLC cells treated with MMC was assessed by measuring nitric oxide metabolites, nitrite and nitrate ($NO_x$) [22]. SCLC cells ($4 \times 10^4$ cells/200 μl) were treated with MMC for 24 h at 37 °C. Nitrate in the culture medium was reduced to nitrite by incubation with nitrate reductase (500 mU/ml), 160 μM NADPH, and 4 μM FAD at room temperature for 2 h. The medium were mixed with an equal amount of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% phosphoric acid). Absorbance was measured at 550 nm, and the amount of nitrite produced was determined using sodium nitrite as the standard. The results were expressed as total nitrite equivalents ($NO_x$).

### 2.12. Measurement of malondialdehyde and carbonyl groups

Lipid peroxidation products of PC12 cells were assessed by measuring the malondialdehyde chromogen formation using thiobarbituric acid [23]. SCLC cells ($2 \times 10^6$ cells/ml) suspended in RPMI medium were treated with MMC for 24 h at 37 °C. Absorbance was measured at 532 nm. The malondialdehyde concentration was expressed as thiobarbituric acid reactive substances (TBARS) nmol/mg of protein using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

The protein oxidation products formed in SCLC cells exposed to MMC for 24 h at 37 °C were quantified by

carbonyl assay using 2,4-dinitrophenylhydrazine [24]. The reaction mixtures incubated with 2 mM 2,4-dinitrophenylhydrazine were sequentially treated with 20% and 10% of trichloroacetic acid. The pellets were washed with the solution (mixtures of ethanol/ethyl acetate mixture (1:1, v/v)) and were dissolved in 2 ml of 6 M guanidine HCl solution. Absorbance was measured at 370 nm, and carbonyls were determined using a molar extinction coefficient of $2.2 \times 10^4\ M^{-1}\ cm^{-1}$.

### 2.13. Statistical analysis

Data are expressed as means ± S.E.M. Statistical analysis was performed by one-way analysis of variance. When significance was detected, post hoc comparisons between the different groups were made using the Duncan's test for multiple comparisons. A probability of less than 0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Cell death and decrease in GSH contents due to MMC

MMC is enzymatically reduced in cells, yielding quinones that generate radicals through redox cycling as well as DNA adducts [1,25]. MMC caused cell death in human SCLC cells in a dose-dependent manner (Fig. 1A). MMC-induced cell death in SCLC cells, approximately correlated with decrease in cellular GSH contents. The present study examined the influence of enzymatic reduction in the cytotoxicity of MMC using Tempol as an inhibitor of the one-electron reduction pathway [26], and dicumarol as an inhibitor of DT-diaphorase inhibitor [27]. Tempol showed a cytotoxicity, nevertheless the presence of Tempol (1–5 mM) inhibited MMC-induced cell death and decrease in the GSH contents in SCLC cells (Fig. 1B, Fig. 2A). Dicumarol (10–40 μM) induced cell death and decrease in the GSH contents, but the cytotoxicity due to MMC plus dicumarol was less than the sum of the each effect of MMC and dicumarol (Fig. 1C, Fig. 2B). The enzymatic reduction of MMC can be catalyzed by xanthine:oxygen oxidoreductase (EC 1,2,3,2; xanthine oxidase) [2]. We examined the effect of xanthine oxidase on cytotoxicity of MMC. The toxic effect of MMC was significantly increased by the addition of 13.4 mU xanthine oxidase (Fig. 1D). Treatment with 1 mM thiol compounds (*N*-acetylcysteine, MPG and L-cysteine) significantly attenuated the enhanced cytotoxicity of MMC due to xanthine oxidase.

The mechanism by which MMC shows toxicity against SCLC cells was investigated. Cell death due to 15 μg/ml MMC was inhibited by 40 μM of z-DQMD.fmk (a cell permeable inhibitor of caspase-3), z-IETD.fmk (a cell permeable inhibitor of caspase-8) and z-LEHD.fmk (a cell permeable inhibitor of caspase-9) (Fig. 3A). Caspase inhibitors at 40 μM alone did not show any cytotoxic effect. One millimole thiol compounds (*N*-acetylcysteine, dithiothreitol and MPG), 100 μM melatonin (a scavenger of reactive oxygen and nitrogen species), 50 μM rutin (the scavenger of nitric oxide and inhibitor of lipid peroxidation) and 25 μM carboxy-PTIO (a scavenger of nitric oxide) decreased cell death due to MMC (Fig. 3B). Decrease in the cellular GSH levels has been shown to increase the sensitivity of cells to damaging effect of various oxidants [12]. Like the effect on cell death, caspase inhibitors (z-DQMD.fmk, z-IETD.fmk and z-LEHD.fmk) and antioxidants (*N*-acetylcysteine, melatonin, rutin and carboxy-PTIO) interfered with decrease in the GSH contents due to MMC (Fig. 3C).

### 3.2. Mitochondrial membrane potential loss, cytochrome c release and caspase-3 activation due to MMC

To assess apoptotic cell death due to MMC and clarify the inhibitory effect of thiol compound on cytotoxicity of MMC, we investigated the nuclear morphological changes in MMC-treated cells. Nuclear staining with Hoechst 33258 demonstrated that control SCLC cells had regular- and round-shaped nuclei. In contrast, the condensation and fragmentation of nuclei, characteristic of apoptotic cells, were evident in SCLC cells treated with 15 μg/ml MMC for 24 h at 37 °C (Fig. 4A). One millimole *N*-acetylcysteine depressed the MMC-induced nuclear damage, while the nuclear morphology in cells treated with thiol compound alone was similar to that in the control cells.

During the process of apoptosis, DNA fragmentation is caused by activation of endonucleases. Fragmented DNA was assessed by measuring the binding of dNTP to the 3′ ends of DNA fragments, which was detected by quantitative colorimetric assay. SCLC cells ($1 \times 10^5$ cells/ml) were treated with 15 μg/ml MMC in the presence of *N*-acetylcysteine for 24 h at 37 °C. The control cells show 0.227 ± 0.014 of absorbance ($n = 6$). Treatment with 15 μg/ml MMC for 24 h caused about 2.5-fold increase in absorbance (Fig. 4B). *N*-acetylcysteine (1 mM) depressed the MMC-induced increase in absorbance, while absorbance in cells treated with *N*-acetylcysteine alone was not significantly different from that in the control cells.

By investigating the effect on the mitochondrial membrane permeability, we assessed the cytotoxic effect of MMC. Opening of the mitochondrial membrane permeability transition pore as an early phenomenon in apoptotic cell death causes a release of cytochrome *c* from mitochondria into the cytosol, leading to the activation of caspases [6]. Change in the mitochondrial transmembrane potential in SCLC cells treated with MMC was quantified by flow cytometry using the cationic lipophilic dye $DiOC_6(3)$. When SCLC cells were treated with 15 μg/ml MMC for 4 h at 37 °C, the increase in the percentage of depolarized cells (characterized by low values of the

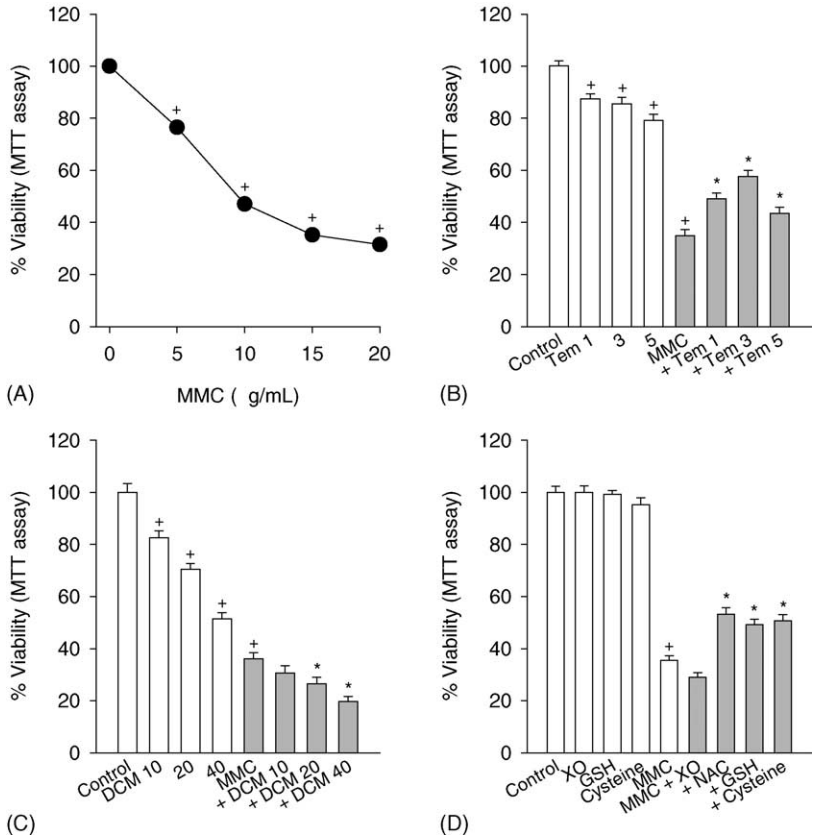


Fig. 1. Cell death due to enzymatic activation of MMC. SCLC cells ($4 \times 10^4$) were treated (A) with various concentrations of MMC (μg/ml), (B) with 15 μg/ml MMC and 1–5 mM Tempol (Tem), or (C) with 15 μg/ml MMC and 10–40 μM dicumarol (DCM) for 24 h at 37 °C. In the experiment of (D), SCLC cells were treated with 15 μg/ml MMC and 13.4 mU xanthine oxidase (XO) in the presence of 1 mM thiol compounds [*N*-acetylcysteine (NAC), GSH and L-cysteine] for 24 h at 37 °C. Data represent means ± S.E.M. of six to eight replicate values in two separate experiments. ($^+$) $P < 0.05$, significantly different from control (percentage of control); ($^*$) $P < 0.05$, significantly different from either MMC alone or MMC plus xanthine oxidase.

transmembrane potential) was observed. One millimole *N*-acetylcysteine depressed the MMC-induced increase in depolarized cells, while the transmembrane potential in the presence of *N*-acetylcysteine alone was similar to that in the control (Fig. 4C).

The MMC-induced cell death was assessed by measuring a release of cytochrome *c* into the cytosol and subsequent activation of caspase-3. Treatment of SCLC cells with 15 μg/ml MMC for 4 h showed a significant increase in cytochrome *c* release (Fig. 5A). One millimole *N*-acetylcysteine, 100 μM melatonin and 50 μM rutin attenuated the MMC-induced release of cytochrome *c*.

Control SCLC cells had caspase-3 activity of 0.834 ± 0.064 U in $2 \times 10^6$ cells. The activity of caspase-3 in SCLC cells treated with 15 μg/ml MMC for 24 h increased to 2.473 U/$2 \times 10^6$ cells. Treatment with 1 mM *N*-acetylcysteine, 1 mM MPG, 100 μM melatonin and 50 μM rutin significantly inhibited the caspase-3 activation due to MMC (Fig. 5B).

### 3.3. Effect of GSH depletion on cytotoxicity of MMC

The present study assessed change in the ROS formation and GSH contents with time, in SCLC cells exposed to MMC. The production of ROS within cells was determined by monitoring conversion of $DCFH_2$-DA to DCF. We examined the formation of ROS and depletion of GSH due to the enzymatic reduction of MMC. Tempol (3 mM) and 20 μM dicumarol induced the formation of ROS and decreased the GSH contents in SCLC cells. Nevertheless, the toxic effect of MMC in the presence of Tempol or dicumarol was less than the sum of the each effect of MMC and Tempol, or of MMC and dicumarol (Fig. 6). The DCF fluorescence in SCLC cells was slowly increased by the addition of 15 μg/ml MMC, and after 4 h of the treatment, the DCF fluorescence significantly increased with time (Fig. 6A). Compared to the ROS formation, the significant decrease in the GSH contents was detected at a 2-h post addition of 15 μg/ml MMC (Fig. 6B).

We investigated the effect of GSH depletion on the cytotoxicity of MMC. SCLC cells were treated with 500 μM L-buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase, for 24 h, and then cells were treated with 15 μg/ml MMC for 6 h. L-buthionine sulfoximine for a 30-h treatment decreased the GSH contents in SCLC cells by 57%. However, this treatment did not significantly induce formation of ROS, caspase-3 activation and cell death (Fig. 7A–C). In this experimental

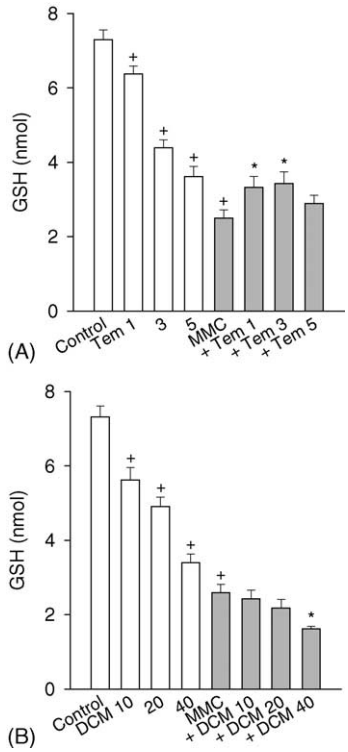


Fig. 2. GSH depletion due to enzymatic activation of MMC. SCLC cells ($4 \times 10^4$) were treated (A) with 15 μg/ml MMC and 1–5 mM Tempol (Tem), or (B) with 15 μg/ml MMC and 10–40 μM dicumarol (DCM) for 24 h at 37 °C. Data are expressed as nmol in GSH contents and represent means ± S.E.M. of six replicate values in two separate experiments. (+) $P < 0.05$, significantly different from control; (*) $P < 0.05$, significantly different from MMC alone.

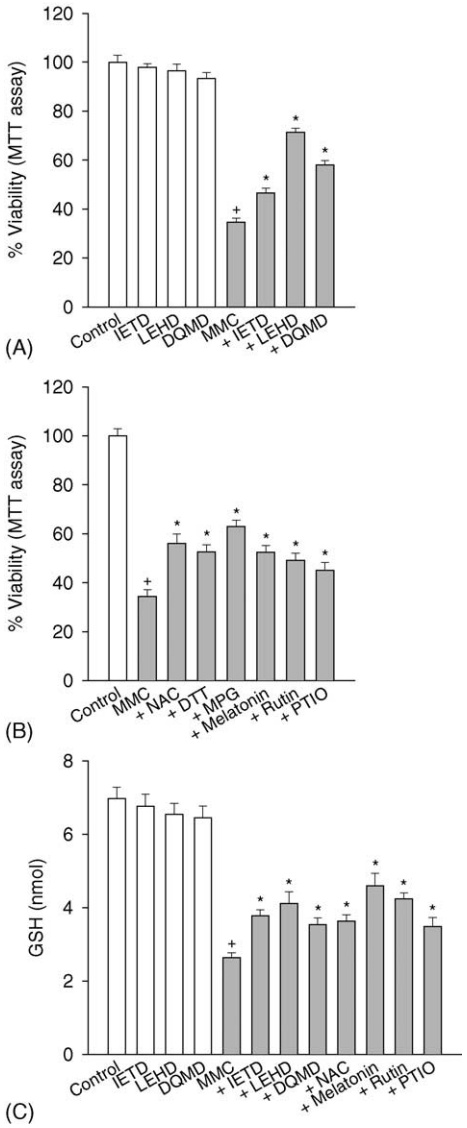


Fig. 3. Cytotoxicity of MMC mediated by caspases. (A, C) SCLC cells ($4 \times 10^4$) were treated with 15 μg/ml MMC in the presence of caspase inhibitors (40 μM of z-IETD.fmk, z-LEHD.fmk and z-DQMD.fmk) or (B) with various antioxidants [1 mM *N*-acetylcysteine (NAC), 1 mM dithiothreitol (DTT), 1 mM MPG, 100 μM melatonin, 50 μM rutin and 25 μM carboxy-PTIO (PTIO)] for 24 h at 37 °C. Data are expressed as percentage of cell viability in MTT assay and nmol in GSH contents. Each point is means ± S.E.M.of six replicate values in two separate experiments. (+) $P < 0.05$, significantly different from control; (*) $P < 0.05$, significantly different from MMC alone.

condition, L-buthionine sulfoximine enhanced the MMC-induced activation of caspase-3 and cell death (Fig. 7A and B). The formation of ROS due to MMC in the presence of L-buthionine sulfoximine was slightly greater than that of MMC alone, but which had no statistical difference. Treatment with 1 mM *N*-acetylcysteine prevented the depletion of GSH due to L-buthionine sulfoximine and attenuated the enhanced ROS formation, caspase-3 activation and cell death due to MMC plus L-buthionine sulfoximine in SCLC cells.

### *3.4. Formation of nitric oxide and oxidation products in SCLC cells treated with MMC*

We examined the formation of nitric oxide in SCLC cells exposed to MMC. In the present study, nitric oxide produced was assessed by measuring nitric oxide metabolites ($NO_x$). The amount of $NO_x$ in intact SCLC cells ($4 \times 10^4$) was 0.66 ± 0.12 μM ($n = 6$). MMC showed a maximum increase in the $NO_x$ production in SCLC cells at 10 μg/ml (2.97 ± 0.22 μM), while beyond this concentration the increasing effect was declined. Decrease in the production of nitric oxide at high concentrations may be ascribed to the cytotoxic effect. The $NO_x$ production in SCLC cells treated with 10 μg/ml MMC was significantly decreased by 1 mM *N*-acetylcysteine, 100 μM melatonin and 50 μM rutin (Fig. 8A). Damage of cell components due to MMC was assessed by measuring formations of malondialdehyde as product of lipid peroxidation, and carbonyls as product of protein oxidation. When SCLC cells were treated with MMC for 24 h at 37 °C, the formations of malondialdehyde and carbonyl were increased with concentration of MMC (Fig. 8B and C). SCLC cells treated with 15 μg/ml MMC for 24 h at 37 °C, produced 0.43 nmol of malondialdehyde and 1.64 nmol of carbonyls in $2 \times 10^6$ cells, respectively. One millimole *N*-acetylcysteine and 100 μM

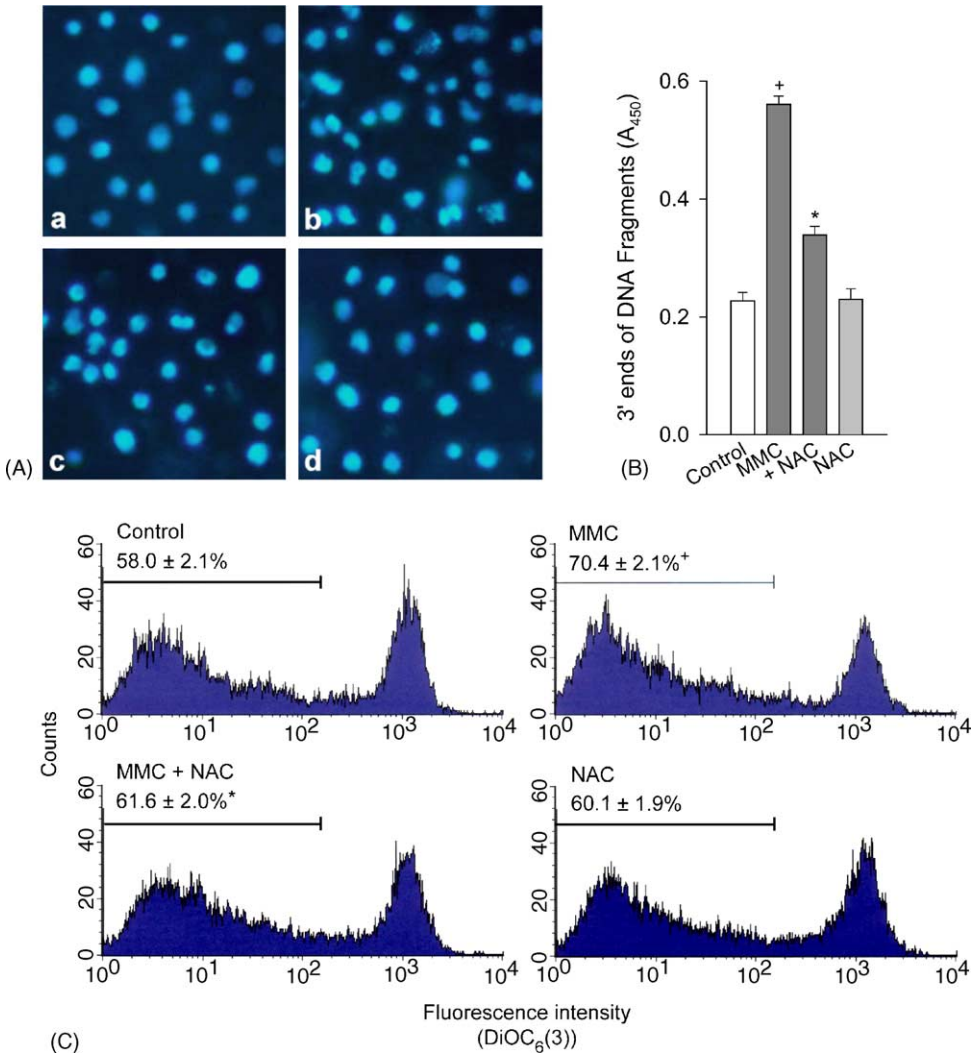


Fig. 4. MMC-induced nuclear damage and loss of the mitochondrial transmembrane potential. In the assay of nuclear damage (A), SCLC cells ($1 \times 10^6$) were treated with 15 μg/ml MMC in the presence of 1 mM *N*-acetylcysteine for 24 h at 37 °C. Cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33258. Figure represents microscopic morphology of the control cells (a), cells treated with MMC alone (b), cells treated with MMC and *N*-acetylcysteine (c) and cells treated with *N*-acetylcysteine alone (d). All the subparts are representative of four different experiments. In the experiment of (B), SCLC cells ($1 \times 10^5$) were treated with 15 μg/ml MMC and 1 mM *N*-acetylcysteine for 24 h at 37 °C. The 3′ ends of DNA fragments were detected as described in Section 2. Data are expressed as absorbance and represent means ± S.E.M. ($n = 6$). In the assay of the mitochondrial transmembrane potential (C), SCLC cells ($1 \times 10^6$) were treated with 15 μg/ml MMC in the presence of 1 mM *N*-acetylcysteine (NAC) for 4 h at 37 °C, and then mixtures were treated with 40 nM $DiOC_6(3)$. Data represent means ± S.E.M. of the percentage-depolarized cells in four independent experiments. ($^+$) $P < 0.05$, significantly different from control; ($^*$) $P < 0.05$, significantly different from MMC alone.

melatonin decreased the formations of malondialdehyde and carbonyls due to MMC (Fig. 8B and C).

## 4. Discussion

The MMC-induced DNA damage and cell death have been shown to be mediated by ROS that are produced during the reduction process of the drug [4,28]. The depressant effects of Tempol, as an inhibitor of the one-electron reduction pathway [26], and of dicumarol, as an inhibitor of DT-diaphorase inhibitor [27], support the fact that cytotoxicity of MMC is mediated by the enzymatic reduction. The enhancement of cytotoxicity due to xanthine oxidase further supports the reductive activation of MMC. The inhibitory effects of antioxidants on the toxicity of MMC, formations of ROS and nitric oxide, and formation of the oxidation products of lipid and protein strongly suggest that the MMC-induced cell death in SCLC cells is caused by oxidative stress. It has been suggested that DNA damage induces the mitochondrial membrane permeability transition [6]. DNA damage due to duocarmycin A may induce the formation of hydrogen peroxide, which causes caspase-3 activation followed by apoptosis [29]. These reports suggest that the free radical-induced DNA damage may be involved in cytotoxicity of MMC.

The ROS formation and GSH depletion due to anticancer drugs may cause mitochondrial dysfunction and

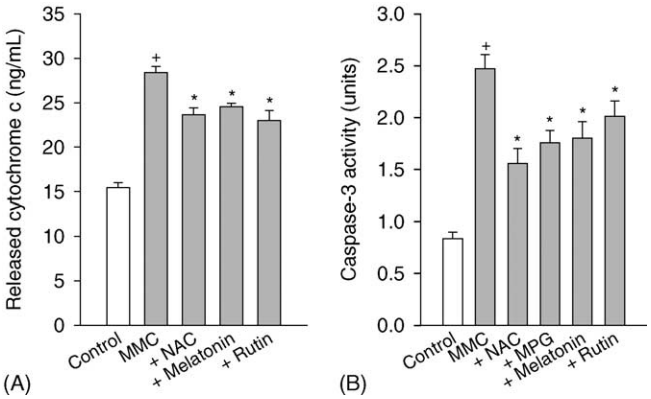


Fig. 5. MMC-induced release of cytochrome *c* and activation of caspase-3. SCLC cells were treated with 15 μg/ml MMC in the presence of various antioxidants [1 mM *N*-acetylcysteine (NAC), 1 mM MPG, 100 μM melatonin and 50 μM rutin]. In the assay of cytochrome *c* (A), SCLC cells ($5 \times 10^5$) were treated with MMC for 4 h at 37 °C, and in the assay of caspase-3 activity (B), $2 \times 10^6$ cells were treated with MMC for 24 h. Data represent means ± S.E.M. of six replicate values in two separate experiments. (+) $P < 0.05$, significantly different from the control; (*) $P < 0.05$, significantly different from MMC alone.

subsequent cytochrome *c* release, which leads to cell viability loss [15,30]. Apoptotic cell death is suggested to be mediated by the interaction of ligand with cell surface CD95 receptor, leading to the activation of caspase-8 by mitochondrial dysfunction, resulting in the release of cytochrome *c* and subsequent activation of caspase-9 and -3 [6,31]. MMC causes disruption of the mitochondrial transmembrane potential in normal human lymphocytes AHH-1, leading to activation of caspase-3 [32]. In contrast to this report, the cytotoxic effect of MMC on human breast cancer MCF-7 cell line is not mediated by the activation of caspase-3 [11]. MMC can induce caspase-8 activation and apoptosis in the absence of CD95 receptor interaction [33]. The mechanism by which MMC causes cell death is therefore uncertain. A significant cytotoxic effect of MMC on SCLC cells was demonstrated by using MTT assay and by observing nuclear morphological changes and increase in DNA fragments. In the present study, the inhibitory effect of bispecific caspase inhibitors (z-DQMD.fmk, z-IETD.fmk and z-LEHD.fmk) suggests that MMC induces apoptotic cell death and depletion of GSH in SCLC cells by both activation of caspase-8 and mitochondrial damage, leading to the activation of caspase-9 and -3.

Loss of the mitochondrial membrane potential causes the release of cytochrome *c* from mitochondria to the cytosol, followed by the activation of caspase-3 that is involved in apoptotic cell death [6]. The condensation and fragmentation of nuclei (Fig. 4) and a significant increase in caspase-3 activity (Fig. 5) were evidence for apoptotic death following exposure to MMC in SCLC cells. It has been suggested that anti-cancer drugs cause cell injury by altering the mitochondrial membrane permeability [29,34]. However, because some anti-cancer drugs cause apoptosis without the cytosolic accumulation of cytochrome *c*, the role of cytochrome *c* in anti-cancer drug-induced cell death has not been clearly elucidated [35]. One of the aims of this study was therefore to clarify whether the cytotoxic effect of MMC is mediated by the mitochondrial membrane permeability change and subsequent release of cytochrome *c*. The present results suggest that the loss of the mitochondrial membrane potential due to MMC causes the release of cytochrome *c* into the cytosol, leading to activation of caspase-9 and -3. This process may elicit apoptotic cell death in SCLC cells. The inhibitory effect of antioxidants, including *N*-acetylcysteine and melatonin, suggests that the MMC-induced cytochrome *c* release and caspase-3 activation appear to be mediated by the formation of free radicals and the GSH depletion.

The oxidation of both GSH and NAD(P)H of mitochondria due to oxidative stress has been suggested to induce the mitochondrial membrane permeability transition [16].

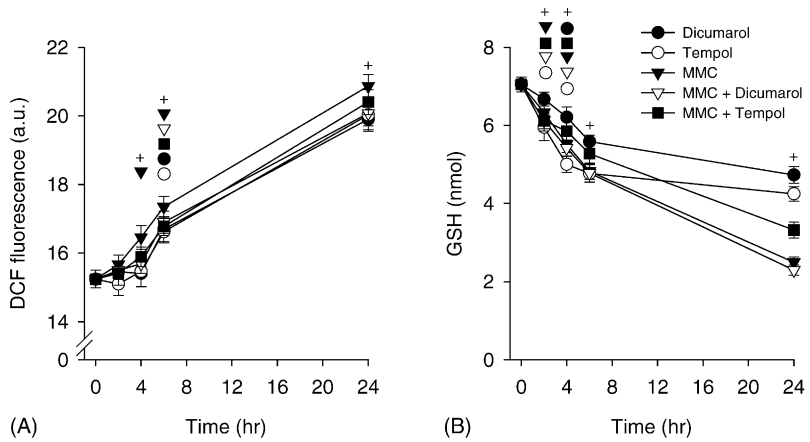


Fig. 6. Change in ROS formation and GSH depletion with time, in cells treated with MMC. SCLC cells ($4 \times 10^4$) were treated with 15 μg/ml MMC in the presence of 3 mM Tempol or 20 μM dicumarol for the stated times at 37 °C, and then the ROS formed and GSH contents were determined. The values are expressed as arbitrary units of fluorescence in ROS formation (A) and nmol in GSH contents (B). Data represent means ± S.E.M. of six replicate values in two separate experiments. (+) $P < 0.05$, significantly different from control.

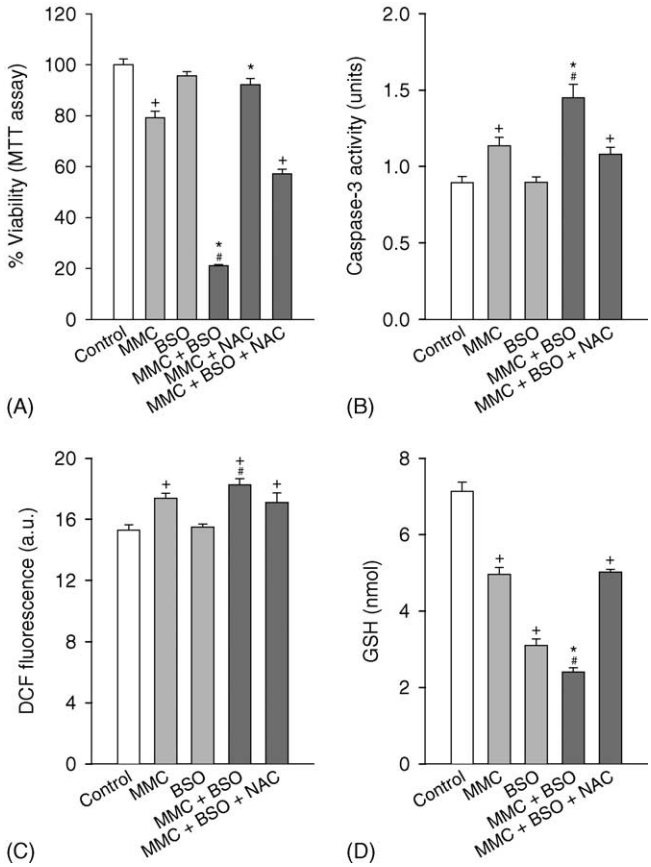


Fig. 7. Increase in cytotoxicity of MMC by GSH depletion. SCLC cells ($4 \times 10^4$) were treated with 500 μM L-buthionine sulfoximine (BSO) for 24 h at 37 °C, and then in this mixture, cells were treated with 15 μg/ml MMC and 1 mM *N*-acetylcysteine (NAC) for 6 h at 37 °C. The values are expressed as the percentage of cell viability (A), units in caspase-3 activity (B), arbitrary units of fluorescence in ROS formation (C), and nmol in GSH contents (D). Data represent means ± S.E.M. of six to eight replicate values in two separate experiments. (+) $P < 0.05$, significantly different from control; (*) $P < 0.05$, significantly different from MMC alone; (#) $P < 0.05$, significantly different from BSO alone.

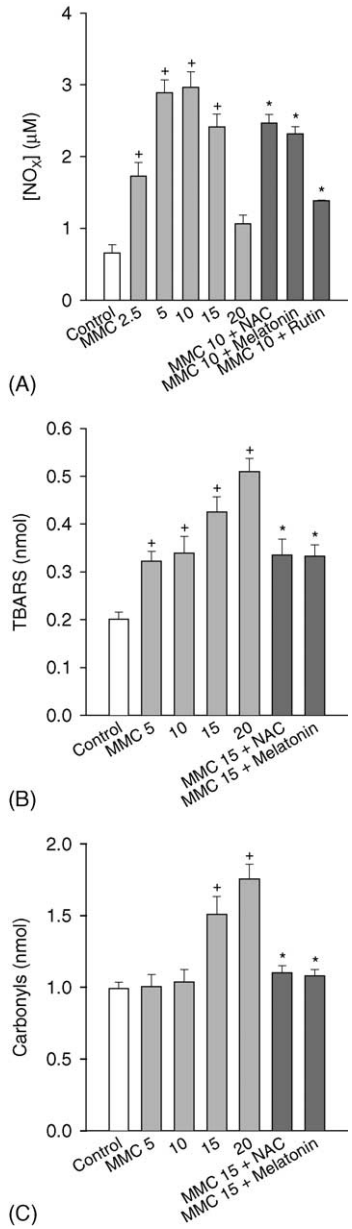


Fig. 8. Formation of nitric oxide and oxidation products in cells treated with MMC. (A) SCLC cells ($4 \times 10^4$) were treated either with various concentrations of MMC (μg/ml) or with 10 μg/ml MMC in the presence of compounds [1 mM *N*-acetylcysteine (NAC), 100 μM melatonin or 50 μM rutin] for 24 h at 37 °C. Nitric oxide metabolites ($NO_x$) were determined. In the experiments of (B) and (C), SCLC cells ($2 \times 10^6$) were treated either with various concentrations of MMC (μg/ml) or with 15 μg/ml MMC in the presence of antioxidants for 24 h at 37 °C. The malondialdehyde (expressed as TBARS) and carbonyls formed were measured. Data are means ± S.E.M. of four to six replicate values in one or two separate experiments. (+) $P < 0.05$, significantly different from control; (*) $P < 0.05$, significantly different from MMC alone.

Drops in GSH levels and concomitant increase in ROS are found during the glutamate-induced apoptotic process in the immortalized hippocampal cell line, HT22 cell [36]. HT22 cells exposed to glutamate show a two-phase increase in ROS production: the initial slow increase in ROS production and the late marked increase in it. Similar to this report, MMC caused a slow increase in the formation of ROS in SCLC cells and showed a significant increase at a 4-h post addition. Meanwhile, MMC markedly decreased the GSH contents in SCLC cells with the treatment time and showed a significant reduction at a 2-h post addition. The degree of GSH depletion approximately correlated with cell death. The present result suggests that the initial depletion of GSH due to MMC in SCLC cells induces the increase in ROS production. Because GSH is the main antioxidant system in cells, the depletion of GSH may predominantly affect cell death due to the late ROS accumulation in SCLC cells treated with MMC. The drops in GSH levels increase the sensitivity of cells to damaging effect of toxic substances [12]. The GSH depletion due to L-buthionine sulfoximine significantly enhanced MMC-induced activation of caspase-3 and cell death, and which was decreased by *N*-acetylcysteine. This result suggests that the toxicity of MMC on SCLC cells is significantly enhanced by the depletion of intracellular GSH level due to formation of ROS.

In conclusion, the results indicate that MMC shows oxidative damage against SCLC cells through the enzymatic reduction. MMC may cause cell death in SCLC cells by inducing the mitochondrial membrane permeability change, leading to caspase-3 activation, and by activation of caspase-8. The toxicity of MMC on SCLC cells appears to be enhanced by the depletion or oxidation of GSH due to oxidative attack of free radicals.

## Acknowledgments

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ1-PG3-20802-0013) to C.S. Lee.

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