Effect of 2-Methoxyestradiol, Buthionine Sulfoximine and Hydrogen Peroxide on the Viability of Renal Carcinoma Cell Lines (ACHN and ACVB)

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2-Methoxyestradiol (2-ME), an endogenous metabolite of 17β-estradiol, induces the intracellular accumulation of superoxide anion (O2·−) and buthionine sulfoximine (BSO) is an inhibitor of glutathione (GSH) synthesis. We have examined the combination anticancer effect of 2-ME and BSO accompanied with hydrogen peroxide (H2O2). 2-ME inhibited cell growth in renal carcinoma cell lines (ACHN and ACVB) accompanied by an increase in the intracellular contents of GSH. The combination of 2-ME, BSO and H2O2 showed a significant antiproliferation effect in both ACHN and ACVB. The intracellular levels of reactive oxygen species (ROS) with a combination with 2-ME and H2O2 in ACHN and ACVB pretreated with BSO were markedly increased, which may have contributed to the potential antiproliferative action.

Key words 2-methoxyestradiol; reactive oxygen species; buthionine sulfoximine; renal carcinoma cell

Reactive oxygen species (ROS) are constantly generated in cells by several biological metabolic processes. A moderate level of intracellular ROS is thought to be important as a stimulant of cellular proliferation. However, the overproduction of ROS can result in detrimental cellular damage, including lipid peroxidation, DNA damage, protein oxidation and enzyme inactivation, which can ultimately lead to cell death.1,2 Aerobic cells contain several antioxidant enzymes, such as superoxide dismutase (SOD), catalase and various peroxidases, to maintain appropriate intracellular ROS levels and to prevent against oxidative damage. Generally, the cellular SOD activity appeared to be a little lower in malignant tissues than in normal tissues.3

2-Methoxyestradiol (2-ME), an endogenous metabolite of 17β-estradiol, is present in human blood and urine. 2-ME is produced by 2-hydroxylation and subsequent O-methylation4 and is known to have a low binding activity to the estrogen receptor.5 2-ME has been shown to have potent anti-proliferative effects on various types of human cancer cells, including leukemia, multiple myeloma, prostate cancer, gastric carcinoma and hepatoma.6–10 2-ME has been reported to induce an accumulation of O2·− by inhibiting SOD, and to make G2/M-phase arrest accompanied by the inhibition of tubulin polymerization.5–10 This compound is currently in clinical trial in the United States.5 Buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis, has been used in clinical trials of cancer treatment.11,12

However, the combination of the effect of 2-ME and the modification of ROS generation and the metabolic system have not been investigated in renal carcinoma cell lines.

Renal carcinoma is one of the drug resistant malignancies in humans. ACHN is a cell line of human renal cell carcinoma, and ACVB is a vinblastine-resistant cell line of ACHN. ACHN has been used to study cytotoxic effects of several anticancer agents. For example, synergistic cytotoxicity by the combination of anti-Fas monoclonal antibody with adriamycin in ACHN had been reported.13 In the present study, we examined the effect of combination with 2-ME, BSO and hydrogen peroxide (H2O2) on the viability of human renal carcinoma cell lines (ACHN and ACVB). We have also analyzed the relationship between cell viability and intracellular ROS levels.

MATERIALS AND METHODS

Chemicals 2-ME, estradiol, dihydroethidium (HEt), 2’,7’-dichlorofluorescin diacetate (DCFH-DA) and buthionine sulfoximine were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). RPMI-1640 was obtained from Invitrogen (Rockvill, MD, U.S.A.). Vinblastine, H2O2 and 5’-5’-dithio-bis 2-nitrobenzoic acid (DTNB) were obtained from Wako Pure Chemicals (Osaka). A WST-1 cell counting kit was obtained from Dojindo (Kumamoto). Fetal bovine serum (FBS) was obtained from ICN (Aurora, OH, U.S.A.).

Cell Line and Cell Culture Human renal carcinoma cell line (ACHN) and its vinblastine resistant cell line (ACVB) were received from Prof. Hirai of Kobe Pharmaceutical University. ACHN was cultured in RPMI-1640 containing 10% FBS, ACVB was cultured with 35 nm vinblastine in RPMI-1640 containing 10% FBS. Cells were routinely grown in an atmosphere of 5% CO2 and 95% air at 37°C.

Growth Inhibition Assay The effect of drugs on cell growth was assayed using the WST-1 method.14 Cells were seeded in 96-well plates, and cultured for 24 h in the presence or the absence of BSO. The cells were then placed in serum-free medium treated with 2-ME and H2O2 in the presence or the absence of BSO for 72 h, after which 10 μl of WST-1 solution was added and incubation was continued for 2 h. The cell absorbance was measured with a microplate reader (Bio-Rad, Hercules, CA, U.S.A.) at 450 nm.

Determination of ROS To measure the level of superoxide anion (O2·−), HEt was used. O2·− oxidizes HEt to ethidium (Et). DCFH-DA was used to measure the level of H2O2.15 H2O2 oxidizes DCFH-DA to 2’,7’-dichlorofluorescein (DCF). After the treatment with drugs, the cells were incubated with HEt (5 μm) and DCFH-DA (5 μm) for 30 min at 37°C. Then, cells were washed with phosphate-buffered saline, harvested and used immediately for flow cytometry analysis. All flow data were analyzed using the CellQuest software package (Becton Dickinson). A total 10000 events...
were collected per test sample; the mean fluorescence intensity obtained is an average value for the fluorescence obtained.

**Determination Total Glutathione (GSH)**  The content of total GSH was determined using Tietze’s method. Cells harvested after being treated with 2-ME for 24 h were homogenized and deproteinized with 5% trichloroacetic acid, followed by neutralization with NaOH. After centrifugation, the supernatant, glutathione reductase, NADPH and DTNB were mixed in a cuvette and the color change was monitored spectrophotometrically at 412 nm.

**Statistical Analysis**  Data are presented as the mean±S.D. Statistical analysis was performed by ANOVA.

RESULTS

Cell viability was reduced to 40% of the control by 72 h treatment with 2-ME at 4 μM for ACHN and ACVB in a concentration-dependent manner. The mean IC \(_{50}\) (inhibitory concentration at 50% viability) value for 2-ME in ACHN and ACVB were 2.6±0.8 μM and 2.2±0.7 μM, respectively. Estradiol had a nonsignificant effect on the viability of ACHN and ACVB up to 10 μM. Amorino et al. \(^{17}\) reported that 2-ME was more cytotoxic than estradiol to H460 human lung carcinoma. This assay confirmed that the endogenous estrogen metabolite, 2-ME, was more effective than estradiol in the growth inhibition of renal carcinoma cells (Fig. 1).

Huang et al. \(^{19}\) reported that 2-ME acts as an anticancer drug by inhibiting SOD activity. We investigated the alteration of intracellular ROS with flow cytometry analysis using two probes. When ACHN and ACVB were treated with 3 μM 2-ME for 24 h, the intracellular O\(_2^-\) and H\(_2\)O\(_2\) levels in ACHN and ACVB were increased (Fig. 2). GSH is a principal antioxidant in the cell, and Dimitrova et al. \(^{18}\) reported that estrogen increased the intracellular GSH levels after treatment with 17β-estradiol. We investigated the intracellular total GSH levels in ACHN and ACVB treated 2-ME. The values of intracellular total GSH treated with 3 μM 2-ME increased about 2-fold in ACHN and 5-fold in ACVB compared with the control. After the treatment with 30 μM estradiol, the GSH contents increased about 2-fold in ACHN and 3-fold in ACVB (Fig. 3).

BSO was reported to decrease the GSH level by inhibiting γ-glutamylcysteine synthetase (γ-GCS). \(^{11}\) BSO induced cytotoxicity by the combination of arsenic trioxide (As\(_2\)O\(_3\)) in renal carcinoma cells. \(^{19}\) The intracellular total GSH contents in ACHN and ACVB were decreased to about 20% after treatment with BSO for 24 h, in comparison with the control (data not shown). The viability of ACHN after 72 h treatment with BSO at 100 μM was reduced by 15%. The viability of ACVB after 72 h treatment with BSO at less than 200 μM was not altered.

Hydrogen peroxide induced concentration-dependent growth inhibition in ACHN and ACVB. The IC \(_{50}\) value for H\(_2\)O\(_2\) in ACHN and ACVB were 9.8±2.7 μM and 10.1±3.8 μM, respectively (data not shown).

Next, we investigated the combined growth inhibitory activity of 2-ME, H\(_2\)O\(_2\) and BSO in ACHN and ACVB. The combined growth inhibition of ROS (H\(_2\)O\(_2\), 7.5 μM) with BSO (25 μM for ACHN, 200 μM for ACVB) and 2-ME (0.1 μM) at sublethal concentrations in ACHN and ACVB was investigated. A significant potentiation of cytotoxicity was obtained when the cells were exposed to 2-ME, H\(_2\)O\(_2\) and BSO. ACVB was observed to be more lethal with this combination treatment than ACHN (Fig. 4).

The changes in intracellular ROS levels after the combination treatment were studied using flow cytometry analysis. As shown in Fig. 5, the percentage in the upper right region, which indicates the fraction of cells with enhanced levels of both O\(_2^-\) and H\(_2\)O\(_2\), were increased from 2.4% to 25% after the combination treatment with 2-ME, H\(_2\)O\(_2\) and BSO.

In Fig. 6, the increase in the percentage of cells with high levels of O\(_2^-\) and H\(_2\)O\(_2\) showed the reduced viability of
was much more cytotoxic than 17β-estradiol, as observed by Oda et al.\textsuperscript{20} The IC\textsubscript{50} of 2-ME for ACHN was comparable to that for ACVB. Chauhan et al.\textsuperscript{21} reported that 2-ME inhibited the growth of multiple myeloma and doxorubicin-resistant multiple myeloma. LaVallee et al.\textsuperscript{22} reported that a metabolite of 2-ME, 2-hydroxyestradiol, was responsible for the proliferative activity. When the concentration was increased to more than 10 μM, the viability was not reduced (Fig. 1). One of the reasons is considered to be the metabolism of 2-ME to 2-hydroxyestradiol.

An increase in the O\textsuperscript{2−} and H\textsubscript{2}O\textsubscript{2} levels were found in the 2-ME treated ACHN and ACVB (Fig. 2). This finding is in agreement with the data of Lin et al.\textsuperscript{7} who reported that 2-ME increased the intracellular O\textsuperscript{2−} and H\textsubscript{2}O\textsubscript{2} levels and induced apoptosis in the gastric carcinoma cell line. And we observed an increase in the intracellular total GSH contents after being treated with 2-ME (Fig. 3). Dimitrova et al.\textsuperscript{18} reported that the intracellular total GSH contents in cultured endothelial cells treated with estradiol was higher than the value in the control. However, the relationship between intracellular total GSH and reactive oxygen species (O\textsuperscript{2−} and H\textsubscript{2}O\textsubscript{2}) levels had remained unknown. Shi et al.\textsuperscript{23} reported that quinone-induced oxidative stress leads to increased γ-GCS activities and GSH synthesis, using rat lung epithelial L2 cells. Therefore, the increase of the total intracellular GSH contents may be the result of γ-GCS activation.

The intracellular total GSH contents in ACHN and ACVB were decreased by BSO, and cell viability was not affected by BSO at 25 μM for ACHN and 200 μM for ACVB. Arsenic trioxide (As\textsubscript{2}O\textsubscript{3}) has been confirmed to be very effective for treating acute promyelocytic leukaemia.\textsuperscript{24} Recently, Wu et al.\textsuperscript{19} reported that BSO in combination with As\textsubscript{2}O\textsubscript{3} had a synergistic inhibitory effect on the proliferation of ACHN. ACVB is a vinblastine-resistant cell line of ACHN. Therefore, we investigated whether or not the cytotoxic effect of 2-ME for ACVB was enhanced by BSO, which inhibits GSH synthesis.

Hydrogen peroxide induced a concentration-dependent cytotoxicity for ACVB as well as ACHN. The viability of treatment by combination with 2-ME, H\textsubscript{2}O\textsubscript{2} and BSO was significantly decreased compared with treatment by 2-ME and H\textsubscript{2}O\textsubscript{2} as well as 2-ME and BSO (Fig. 4). The intracellular O\textsuperscript{2−} and H\textsubscript{2}O\textsubscript{2} levels were increased significantly when treated with a combination of 2-ME, H\textsubscript{2}O\textsubscript{2} and BSO compared with 2-ME and H\textsubscript{2}O\textsubscript{2} (Fig. 5). The correlation between the percentage of cells with enhanced levels of O\textsuperscript{2−} and H\textsubscript{2}O\textsubscript{2} and the cell viability of ACVB suggest that the increase of intracellular O\textsuperscript{2−} and H\textsubscript{2}O\textsubscript{2} levels may contribute to the decrease in viability (Fig. 6). Lambert et al.\textsuperscript{25} reported an inhibition of the viability using 2-ME and ROS-generating treatment (hypoxanthine/xanthine oxidase). Wu et al.\textsuperscript{19} reported the enhancement of the As\textsubscript{2}O\textsubscript{3}-induced cytotoxicity by BSO in ACHN. We observed an enhanced cytotoxicity with the combination of BSO, H\textsubscript{2}O\textsubscript{2} and 2-ME at a nonlethal concentration.

In recent years, it has become apparent that mitochondria play a critical role in the mechanism of both apoptosis and necrotic cell death. The effect of 2-ME on intracellular ROS production was considered to be involved in the inhibition of mitochondrial respiration.\textsuperscript{26} Felty and Roy\textsuperscript{27} reported that estrogens stimulate rapid production of intracellular ROS,
which leads to the phosphorylation of c-jun and CREB, and increased activity of redox sensitive transcription factors, NRF-1, c-jun and CREB, known to be involved in the regulation of cell cycle genes for estrogen-dependent cells. We observed an increase in the O$_2^-$ and H$_2$O$_2$ levels for ACHN and ACVB treated with 2-ME (3 μM) (Fig. 2).

In summary, the present study shows that 2-ME can increase the intracellular ROS production in a renal carcinoma cell line. We observed that the treatment of 2-ME, at a non-lethal concentration, combination with the modification of the antioxidant system by BSO, resulted in an increase of the intracellular ROS levels and showed an enhanced growth inhibition activity.

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