Introduction

Arsenic is a natural substance that has been used medicinally for over 2400 years. Arsenic was rekindled after it was identified as an active ingredient in traditional medicines in China. Arsenic drugs have been generally used for the treatment of malignant hematologic diseases. Arsenic trioxide (As$_2$O$_3$) has been confirmed to be an effective treatment for acute promyelocytic leukemia (APL). It has been reported that the effects of As$_2$O$_3$ are not confined to APL cells but can also be observed in various myeloid and lymphoid cells. The action mechanisms of As$_2$O$_3$ in APL and other malignancies are thought to involve inhibition of growth and induction of apoptosis.

MOLT-4, a human T-lymphoblastoid leukemia cell line, has been used extensively for studies of leukemia cell biology and antileukemia therapy. We have established a daunorubicin-resistant MOLT-4 subline MOLT-4/DNR by exposing the parental MOLT-4 cells stepwise to increasing concentrations of daunorubicin. MOLT-4/DNR cells overexpress functional P-glycoprotein and MDR1 mRNA. Most of the drugs excreted via this efflux pump are hydrophobic organic compounds, and As$_2$O$_3$ may not be excluded from drug-resistant cells expressing functional P-glycoprotein. However, whether As$_2$O$_3$ affects the growth of lymphocytic leukemia cells expressing functional P-glycoprotein is unknown. Thus, in Chapter 1, the effects of As$_2$O$_3$ on the growth and apoptosis of parental MOLT-4 and the resistant MOLT-4/DNR cells were investigated.

Cancer cell sensitivity to As$_2$O$_3$ correlates with intracellular glutathione
levels. Cells expressing higher levels of glutathione or glutathione-associated enzymes are less sensitive to \( \text{As}_2\text{O}_3 \) than cells expressing lower levels of these molecules. Arsenic-resistant cells are also reported to contain higher levels of glutathione. Moreover, cells with increased glutathione levels can be sensitized to \( \text{As}_2\text{O}_3 \) by agents that deplete intracellular glutathione. Thus, in Chapter 2, the apoptosis-inducing effects of \( \text{As}_2\text{O}_3 \) in the presence of glutathione modulators in MOLT-4 and MOLT-4/DNR cells were examined.

It has been recognized that benefit and risk of arsenic are strictly dependent on the individual chemical forms of arsenic. Although \( \text{As}_2\text{O}_3 \) has been confirmed to be an effective treatment for APL, serious adverse drug reaction induced by \( \text{As}_2\text{O}_3 \) was occasionally reported. Arsenic disulfide (\( \text{As}_2\text{S}_2 \)), the most important component of \textit{Xiong huang}, was a candidate for its good therapeutic reputation and perceived low toxicity in traditional medicines. \textit{Xiong huang} was reported to improve the clinical outcomes of hematologic malignancies in our clinical trials, which could be attributed to \( \text{As}_2\text{S}_2 \). \( \text{As}_2\text{S}_2 \)-mediated growth inhibition and apoptosis induction have been found in leukemia K562 cells and other cancer cells. However, the effects of \( \text{As}_2\text{S}_2 \) on cells of a human APL cell line HL-60 cells with a particular focus on proliferation and differentiation have not been addressed. Thus, in Chapter 3, the effects of \( \text{As}_2\text{S}_2 \) on HL-60 cells were investigated by focusing on differentiation, generation of reactive oxygen species, intracellular glutathione depletion, and activation of p38 MAPK.

Patients with AML from myelodysplastic syndrome (MDS/AML) have higher probabilities of resistance to chemotherapy, lower rates of complete remission, and the poor prognosis. MDS/AML patients tended to have complex type abnormalities including monosomy 7 (-7), which are considered to be an unfavorable risk subgroup. We have revealed that \( \text{As}_2\text{S}_2 \) is effective in the treatment of MDS without serious adverse drug reaction. A leukemic F-36p cell line has been established from a patient diagnosed with refractory anemia with excess blasts. Thus, in Chapter 4, the effective mechanisms of \( \text{As}_2\text{S}_2 \) in the treatment of MDS or MDS/AML were studied by use of F-36p cell line.

\textbf{Chapter 1. Arsenic trioxide induces apoptosis equally in T lymphoblastoid leukemia MOLT-4 cells and P-glycoprotein-expressing daunorubicin-resistant MOLT-4 cells}

\( \text{As}_2\text{O}_3 \) inhibited the growth and survival of MOLT-4 and MOLT-4/DNR cells in a time- and dose dependent manner. \( \text{As}_2\text{O}_3 \) induced apoptotic morphology in both MOLT-4 and MOLT-4/DNR cell lines. These effects were time- and
dose-dependent. \( \text{As}_2\text{O}_3 \) did not change the percentage of P-glycoprotein-expressing cells or the efflux ability of MOLT-4/DNR cells.

Thus, the data in this Chapter showed that \( \text{As}_2\text{O}_3 \) inhibited growth and induced apoptosis equally in MOLT-4 and MOLT-4/DNR cells, and this suppressive effect was not influenced by P-glycoprotein expression or function in MOLT-4/DNR cells.

Chapter 2. Arsenic trioxide induces apoptosis in cells of MOLT-4 and its daunorubicin-resistant cell line via depletion of intracellular glutathione, disruption of mitochondrial membrane potential, and activation of caspase-3

MOLT-4 cells and MOLT-4/DNR cells were similarly sensitive to the apoptosis-inducing effect of \( \text{As}_2\text{O}_3 \). Buthionine sulfoxide (BSO), a selective inhibitor of \( \gamma \)-glutamylcysteine synthetase, and ascorbic acid (AA), having pro-oxidant properties, rendered these cells more sensitive to \( \text{As}_2\text{O}_3 \), whereas N-acetylcysteine (NAC), an antioxidant since it donates a cysteine to the de novo synthesis of glutathione, reduced this sensitivity. BSO and AA decreased, but NAC increased, the intracellular glutathione contents of both MOLT-4 and MOLT-4/DNR cells. Decreasing glutathione with BSO potentiated \( \text{As}_2\text{O}_3 \)-mediated growth inhibition, disruption of mitochondrial membrane potential, activation of caspase-3, and apoptosis of cells. Clinically relevant doses of AA enhanced the anticancer effects of \( \text{As}_2\text{O}_3 \) via the disruption of mitochondrial membrane potential, activation of caspase-3, and induction of apoptosis. In contrast, increase in glutathione levels with NAC attenuated all of these \( \text{As}_2\text{O}_3 \)-mediated actions.

Thus, MOLT-4 and MOLT-4/DNR cell sensitivity to \( \text{As}_2\text{O}_3 \) was associated with the intracellular glutathione content. \( \text{As}_2\text{O}_3 \) induced apoptosis in MOLT-4...
and MOLT-4/DNR cells expressing functional P-glycoprotein via depletion of intracellular glutathione, and subsequent disruption of mitochondrial membrane potential and activation of caspase-3.

Fig. 2 As$_2$O$_3$-induced apoptosis and its modulation by BSO, AA, or NAC in MOLT-4 cells with 5 μM As$_2$O$_3$ alone or with a combination of 5 μM As$_2$O$_3$ and 125 μM AA, 100 μM BSO, or 10 mM NAC for 72 h. Values are the means ± SD of three independent experiments. *p<0.01, **p<0.001 vs control; *p<0.01, **p<0.001 vs As$_2$O$_3$ alone.

Chapter 3. Involvement of oxidative stress associated with glutathione depletion and p38 MAPK activation in arsenic disulfide-induced differentiation in HL-60 cells

As$_2$S$_2$ induced cell differentiation based on the increment in expression of CD11b, antibody specific for the myeloid differentiation marker, nitroblue tetrazolium-positive cells, and cell size. A transient increase in generation of reactive oxygen species level along with intracellular glutathione level was also observed. p38 MAPK activation gradually increased after generation of reactive oxygen species and sustained during the cell differentiation. Decreased CD11b expression was accompanied by p38 MAPK activation, and p38 MAPK inhibitor restored the CD11b expression.

Thus, the data in this Chapter showed that As$_2$S$_2$ induced differentiation in HL-60 cells, and moderate levels of oxidative stress induced by As$_2$S$_2$ positively contribute to HL-60 cell differentiation. The activation of p38 MAPK resulted from oxidative stress seems to be implicated in the negative regulation of the

Fig. 3 The modified effects of inhibitor (SB203580) of p38 MAPK on As$_2$S$_2$-induced differentiation in HL-60 cells. Cells were incubated with 10μM SB203580 (SB) and 8 μM As$_2$S$_2$ (A8) alone or in combination (SB + A8) for 72 h, respectively. After treatment, the cells were
stained for CD11b and then analyzed with flow cytometer. Data are the mean ± SD of 3 independent experiments. Means were compared by 1-way ANOVA. *p< 0.05 as compared with 8 μM As₂S₂ (A8) alone.

Chapter 4. Arsenic disulfide induced apoptosis and concurrently promoted erythroid differentiation in cytokine-dependent MDS-progressed leukemia cell line F-36p with complex karyotype including monosomy 7.

As₂S₂ inhibited the proliferation of F-36p cells. The apoptotic cells significantly increased and were in a dose-dependent manner. The cell viabilities were significantly inhibited by As₂S₂ and were in dose-dependent. Significant increases of expression of CD235a, antibody specific for the erythroid differentiation marker glycophorin A (Gly A), were concurrently observed and were also in a dose-dependent manner.

In this Chapter, I showed that F-36p cell line might provide a desirable cell model for the study of effective mechanisms of As₂S₂ in the treatment of MDS or MDS/AML. As₂S₂ could inhibit proliferation and viability, induce apoptosis, and concurrently promote erythroid differentiation in F-36p cells, which were in a dose-dependent manner.

![Fig. 4 Comparison of levels of CD235a-positive cells.](image)

Conclusions

Effects of As₂O₃ are not confined to APL cells but can also be observed in various other cell lines and in drug-resistant sublines. As₂O₃ induced apoptosis in parent MOLT-4 cells and MOLT-4/DNR cells expressing functional P via depletion of intracellular glutathione, and subsequent disruption of mitochondrial membrane potential and activation of caspase-3. As₂S₂ was a candidate for its good therapeutic reputation and perceived low toxicity in traditional medicines. The data showed that moderate levels of oxidative stress induced by As₂S₂ positively contribute to HL-60 cell differentiation, while the activation of p38 MAPK resulted from oxidative stress seems to be implicated in the negative
regulation of the differentiation. Furthermore, it is the first description that As$_2$S$_2$ can inhibit proliferation and viability, induce apoptosis, and concurrently promote erythroid differentiation in cytokine-dependent MDS-progressed human leukemia cell line F-36p with complex karyotype including karyotype -7. The precise action mechanisms of As$_2$S$_2$ demonstrated in this study in human malignant cells might imply the rationale and future directions of As$_2$S$_2$ as a potential anticancer drug candidate.

**PUBLICATIONS**

論文審査の結果の要旨

亜砒酸（As$_2$O$_3$）に代表される無機砒素化合物は、毒物としてよく知られる反面、各種白血病の治療に有効性を示す報告が多数あり、本邦では急性前骨髄球性白血病の治療薬として適応となっている。中国ではAs$_2$O$_3$の他、より毒性の低いAs$_2$S$_2$も各種白血病の治療に臨床で用いられているが、これら無機砒素化合物の抗白血病作用には不明な点が多く、特にAs$_2$S$_2$に至っては、その作用に関する科学的解析がほとんどなされていない。胡晓梅氏の博士学位論文は、Tリンパ芽球性白血病細胞、急性前骨髄球性白血病細胞、あるいは骨髄異形成症候群から悪性化した急性白血病細胞の各ヒト白血病細胞株を用い、As$_2$O$_3$やAs$_2$S$_2$の抗白血病効果とその作用機序について検討した結果をまとめたものである。

論文は、次の4章に分けて論じている。

第1章では、ヒトTリンパ芽球性白血病MOLT-4細胞株およびP-糖タンパク質を高発現するダウノルビシン耐性MOLT-4細胞（MOLT-4/DNR細胞）株に対する、As$_2$O$_3$の効果を検討した。As$_2$O$_3$は、いずれの細胞株に対しても細胞増殖抑制作用を示し、MOLT-4/DNR細胞に対するAs$_2$O$_3$の効果は、P-糖タンパクとは無関係に発現することから、As$_2$O$_3$が多剤耐性白血病細胞に対しても有効であることを示唆した。

第2章では、MOLT-4およびMOLT-4/DNR細胞株に対するAs$_2$O$_3$の作用機序をより詳細に検討した。As$_2$O$_3$はいずれの細胞株に対しても細胞内グルタチオン濃度を低下させ、その結果細胞のミトコンドリア膜電位を下げ、細胞のアポトーシスを誘導することを示した。

第3章では、ヒト前骨髄球性白血病細胞株であるHL-60細胞株に対するAs$_2$S$_2$の効果を検討した。As$_2$S$_2$はHL-60細胞の赤血球様分化を促すこと、および高レベルの酸化ストレスはp38MAPK活性を上昇させてAs$_2$S$_2$分化誘導作用を阻害することを示した。

第4章では、骨髄異形成症候群から悪性化した急性白血病の細胞株であるF-36P細胞株に対するAs$_2$S$_2$の作用を検討した。As$_2$S$_2$は、F-36P細胞の赤血球様分化を誘導し、またF-36P細胞にアポトーシスを誘導して細胞増殖を抑制することを明らかとした。

以上本論文は、無機砒素化合物のAs$_2$O$_3$やAs$_2$S$_2$が、種々のヒト白血病細胞株に対して細胞増殖抑制効果、アポトーシス誘導作用、および細胞分化誘導作用を示すことを明らかとした。As$_2$O$_3$は、本邦でも急性前骨髄球性白血病の治療に適応となっているが、本研究ではP-糖タンパク質を高発現するダウノルビシン耐性の白
血病細胞に対してもAs₂O₃が有用であることを示した。さらに本研究では、As₂S₂が骨髄異形成症候群由来の白血病に対しても治療効果を有することを示唆した初めての知見と言える。

このように本論文は、種々の興味ある内容を含んでおり、また論文としても良くまとまっていることから、博士（薬学）の学位に十分値するものと判断した。