INTRODUCTION
The medicinal application of arsenic compounds can be dated back more than 2000 years to ancient China and Europe. Over recent decades, accumulating studies have shown the efficacy of arsenics, such as arsenic trioxide (ATO), in the treatment of different types of carcinoma including breast cancer. However, these agents are limited in the clinical applications due to the serious adverse effects arise from their high toxicity. Therefore, alternative agents with similar therapeutic efficacy but fewer adverse effects are urgently required to be developed. Arsenic disulfide (As$_2$S$_2$), the main active ingredient of orange-red crystalline realgar, also known as ‘Xiong-Huang’ in traditional Chinese medicine, was chosen to be a candidate for its good therapeutic reputation and relatively low toxicity in the treatment of various types of malignancies. In recent years, clinical trials have shown the promising anticancer efficacies of As$_2$S$_2$ in treatment of refractory or relapsed acute promyelocytic leukemia (APL) and chronic myelogenous leukemia (CML) in China. Besides, experimental researches suggested therapeutic effects of As$_2$S$_2$ on various solid tumors. Nevertheless, a limited number of studies have reported the cytotoxic effect of As$_2$S$_2$ on human breast cancer. In addition, there are rare cases of studies investigating the underlying mechanisms of the effects of As$_2$S$_2$ against breast carcinoma.

The present study aimed to investigate: (1) the effects of As$_2$S$_2$ on human breast cancer cell lines and (2) the possible molecular mechanisms underlying the action of the drug, as well as (3) the synergistic anticancer effects of As$_2$S$_2$ combined with L-buthionine-(S, R)-sulfoximine (BSO) on breast cancer cells.

CHAPTER ONE  Anticancer efficacies and underlying mechanism of action of arsenic disulfide on breast cancer cells in different culture systems
Recent studies suggest that growing cancer cells in three dimensional (3D) cell culture system mimic more closely the in vivo environment compared with traditional two dimensional (2D)
cell culture system. The effects of As$_2$S$_2$ on human breast cancer MCF-7 cells were compared between 2D and 3D cultures, and the possible molecular mechanisms underlying the action of this drug were studied. The results showed that As$_2$S$_2$ inhibited viability of MCF-7 cells and induced cell apoptosis in both 2D monolayers and 3D spheroids in a concentration dependent manner (Figure 1). In addition, the cytotoxic selectivity of As$_2$S$_2$ on MCF-7 cells in comparison with normal human breast epithelial cells (184B5) was investigated. As$_2$S$_2$ exerted a cytotoxic action in both MCF-7 and 184B5 cells in a dose-dependent manner. However, in comparison with MCF-7 cells, 184B5 cells were less sensitive to the cytotoxic effects of As$_2$S$_2$ (Table 1), indicating the cytotoxic selectivity of As$_2$S$_2$ to human breast cancer cells as compared to normal human breast epithelial cells. Furthermore, observations obtained from a three-dimensional (3D) cultured system provided more convincing evidence to confirm the cytotoxic selectivity of As$_2$S$_2$ in breast cancer cells but not in normal breast epithelial cells. This chapter thus provided an experimental confirmation for the cytotoxicity of As$_2$S$_2$ against breast cancer cells and the drug safety of As$_2$S$_2$ as a relatively less toxic agent to normal breast cells.

Figure 1 Effects of As$_2$S$_2$ on viability of MCF-7 cells in 2D (open symbol) and 3D (closed symbol) culture systems. Cells were cultured with As$_2$S$_2$ for 72h, and the cell viability was assessed by CCK-8 assay. (A) Cell viability of MCF-7 monolayers (2D) and spheroids (3D). (B) Dose response curves for the inhibitory effects of As$_2$S$_2$ on the cell viability of 2D-cultured (○) and 3D-cultured (●) MCF-7 cells. (C) The mean IC$_{50}$ values of As$_2$S$_2$ on MCF-7 cell viabilities in 2D and 3D culture systems. Asterisks indicate significant differences between control and drug treatment groups (**$P < 0.01$ and ****$P < 0.0001$). Hashes indicate significant differences between 2D and 3D cultured cells (##$P < 0.01$ and ###$P < 0.001$).

Table 1 IC$_{50}$ values of As$_2$S$_2$ on the growth of normal breast and breast cancer cells in 2D- and 3D-cultured systems

<table>
<thead>
<tr>
<th>Cell line</th>
<th>2D</th>
<th>3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>184B5</td>
<td>12.39 ± 0.36</td>
<td>&gt; 16</td>
</tr>
<tr>
<td>MCF-7</td>
<td>5.45 ± 0.36*</td>
<td>8.61 ± 3.09</td>
</tr>
</tbody>
</table>

Abbreviations: As$_2$S$_2$, arsenic disulfide; 2D, two dimensional; 3D, three dimensional. *Significantly low IC$_{50}$ value (high sensitivity) was observed, as compared to the value in 184B5 cells ($P = 0.0002$).

CHAPTER TWO  Anticancer efficacies and underlying mechanism of the action of arsenic disulfide on different breast cancer cells
The anticancer effects of As$_2$S$_2$ on two typically distinctive subtypes of breast cancer cell lines, namely MCF-7 and MDA-MB-231, in vitro (Figure 2) and the possible underlying mechanisms were investigated. The results identified the antitumor effects of As$_2$S$_2$ against breast cancer cells, which included the inhibition of cell proliferation (Figure 2), cell survival, and cell invasion. These effects of As$_2$S$_2$ were associated with blockade of cell cycle progression and induction of apoptosis and autophagy. Inhibition of PI3K/Akt signals (Figure 3), decrease in Matrix metalloproteinase-9 (MMP-9) expression (Figure 4), and cellular reactive oxygen species (ROS) accumulation were also suggested to be implicated in antitumor activities of As$_2$S$_2$.

**Figure 2** As$_2$S$_2$ inhibited viability of cells of the human breast cancer cell lines MCF-7 and MDA-MB-231. MCF-7 and MDA-MB-231 cells were treated with As$_2$S$_2$ for 48 h, and the cell viability was determined by CCK-8 assay. Data are presented as the mean ± SEM (n ≥ 3). *P < 0.05, **P < 0.01 vs. control group (As$_2$S$_2$ 0 µM).

**Figure 3** Effects of As$_2$S$_2$ on the expression of cell survival related-proteins. MCF-7 and MDA-MB-231 cells were treated with 0, 4, 8 and 16 µM As$_2$S$_2$ for 48 h, and Western blot assays were carried out to examine expression of PI3K and Akt. β-actin was used as an internal control. Asterisks indicate significant differences between the control (As$_2$S$_2$ 0 µM) and the As$_2$S$_2$-treated groups (*P < 0.05, **P < 0.01).

**Figure 4** Effects of As$_2$S$_2$ on MMP-9 protein expression in breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with As$_2$S$_2$ for 48 h. Then, Western blot assays were carried out to examine the effects of As$_2$S$_2$ on the expression of MMP-9. β-actin was used as an internal control. Asterisks indicate significant differences between the control cells (As$_2$S$_2$ 0 µM) and As$_2$S$_2$-treated cells (*P < 0.05).

CHAPTER THREE  Synergistic antitumor effects of arsenic disulfide in combination with buthionine sulfoximine on breast cancer cells
Combination chemotherapy represents an effective approach to potentiate the therapeutic efficacy, overcome drug resistance, reduce adverse effects, and minimize drug dosage of each compound alone. In this Chapter, I investigated the synergistic anticancer effects of As$_2$S$_2$ combined with BSO, a potent specific inhibitor of glutathione (GSH) biosynthesis, on human breast cancer MCF-7 cells cultured in both 2D monolayers and 3D spheroids (Figure 5). The results suggested that the combination of As$_2$S$_2$ and BSO synergistically decreased the amount of intracellular GSH and conversely potentiated reactive oxygen toxicity in human breast cancer cells, which resulted in cell cycle arrest (Figure 6), apoptosis induction, and cell survival inhibition. Thus, the combination treatment with As$_2$S$_2$ and BSO might be a promising therapeutic strategy to increase drug sensitivity of breast cancer cells to As$_2$S$_2$, as well as to overcome their drug resistance.

**Figure 5** Changes in Calcein-AM (first line), Hoechst 33342 (second line), and PI (third line) stained morphology induced by As$_2$S$_2$ and BSO in 2D- and 3D-cultured MCF-7 cells. Both MCF-7 monolayers (2D) and spheroids (3D) were treated with 4 µM of As$_2$S$_2$, 1 µM of BSO, and their combination for 72 h. Merging (fourth line) of calcein-AM, Hoechst, and PI fluorescence were shown.

**Figure 6** BSO augments cell cycle arrest triggered by As$_2$S$_2$ in both 2D- and 3D-cultured MCF-7 cells. MCF-7 cells cultured in (A) 2D and (B) 3D systems were treated with 4 µM of As$_2$S$_2$ and 1 µM of BSO, alone or in combination, for 72 h. Percentages of cell numbers in each phase were assessed (lower two figures). All data were expressed as mean ± SEM (n ≥ 3). *P < 0.05, **P < 0.01 vs. control; ***P < 0.01 vs. As$_2$S$_2$ alone.

**CONCLUSION**

The aim of the present study is to investigate the efficacy, toxicity, and mechanisms of As$_2$S$_2$
action in treatment of breast cancer, using 2D- and 3D-cultured human breast cancer cell lines. In addition, the therapeutic efficacy of \( \text{As}_2\text{S}_2 \) combined with BSO was also studied. The results demonstrated that human breast cancer MCF-7 cells were more sensitive to \( \text{As}_2\text{S}_2 \) in comparison with normal breast epithelial cells in both 2D and 3D culture systems, suggesting significant antitumor effects of \( \text{As}_2\text{S}_2 \) on breast carcinoma, which were further characterized by inhibiting cell growth, inducing apoptosis, alleviating cell migration and arresting cell cycle in different breast cancer cell lines. The results also provided insights that the combination application of \( \text{As}_2\text{S}_2 \) with BSO could improve the therapeutic efficacy of \( \text{As}_2\text{S}_2 \) in treatment of drug resistant breast carcinomas.

**PUBLICATIONS**

論文審査の結果の要旨

亜ヒ酸（As₂O₃）に代表される無機ヒ素化合物は、毒物として知られる反面、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As₂O₃の他、より毒性の低いAs₂S₂も各種白血病の治療に臨床で用いられているが、これら無機ヒ素化合物の抗腫瘍効果には不明な点が多く、特にAs₂S₂に至っては、その作用に関する科学的解析がほとんどされていない。またヒ素化合物が、乳癌をはじめとする種々の固形腫瘍に有効であるとする報告がある一方、これらの癌に対するAs₂S₂の効果についてはほとんど報告がない。本論文は、通常の単層培養（2次元培養）系およびスフェロイドを形成させる3次元培養系を用いて培養したヒト乳癌細胞に対する、As₂S₂の効果とその作用機序を検討した結果をまとめたものであり、次の3章に分けて論じている。

第1章では、ヒト乳癌細胞株MCF-7細胞の2次元および3次元培養系を確立し、As₂S₂の増殖抑制効果を比較検討した。2次元および3次元のいずれの培養系においても、As₂S₂はMCF-7細胞の生存率を用量依存的かつ有意に減少させた。さらには、これらの培養系細胞に対するAs₂S₂のアポトーシス誘導作用や、アポトーシス関連タンパク質発現に対するAs₂S₂の有用な効果を確認した。また本章では、2次元培養したMCF-7細胞に比べ3次元培養したMCF-7細胞の方が、As₂S₂の作用に耐性を示すことを明らかとした。

更に本章では、MCF-7細胞および正常乳腺由来細胞株184B5細胞に対するAs₂S₂の効果を比較検討した。As₂S₂が184B5細胞の生存率に及ぼす影響は弱かった。特に3次元培養した184B5細胞の生存率に対しては、ほとんど影響を及ぼさなかった。これらの結果から、As₂S₂はヒト乳癌細胞に特異的に作用し、正常乳腺細胞に対してはほとんど毒性を示さないものと結論した。

第2章では、エストロゲン受容体を有するMCF-7細胞と、ホルモン受容体の無いヒト乳癌細胞株MDA-MB-231細胞を用い、2次元および3次元培養したこれらの細胞の生存率に及ぼすAs₂S₂の作用およびその作用機序を比較検討した。As₂S₂はいずれの培養系においても、両細胞株細胞の生存率を低下させ、また細胞周期の進行を阻害してアポトーシスとオートファジーを誘導した。これらAs₂S₂の効果は、PI3K/Aktシグナルやアポトーシス関連タンパク質への影響、および細胞内活性酸素分子種の蓄積と関連しているものと考えられた。

第3章では、第1章で述べた3次元培養MCF-7細胞のAs₂S₂耐性を克服する戦略として、細胞内グルタチオンレベルを下げる作用のあるDL-buthionine-(S, R)-sulfoximine (BSO)をAs₂S₂と併用したときの効果を検討した。As₂S₂単独の場合と比べ、As₂S₂にBSOを併用した場合には、3次元培養MCF-7細胞の生存率は有意に減少した。As₂S₂とBSOの併用効果は、3次元培養MCF-7細胞に対する細胞周期の進行の阻害、アポトーシスの誘導、および細胞内グルタチオンレベルの減少に関連するものと考えられた。

以上本論文は、無機ヒ素化合物のAs₂S₂が、2次元および3次元培養したヒト乳癌細胞に対し抑制効果を有し、乳癌の治療に有用な候補化合物となり得ることを示した。また本研究ではAs₂S₂の効果に関する分子機序についても検討し、ヒト乳癌細胞に対してAs₂S₂が細胞周期の進行を抑え、アポトーシスやオートファジーを誘導することを明らかとした。さらに本論文では、3次元培養したヒト乳癌細胞が示すAs₂S₂耐性を克服する手段として、BSOをAs₂S₂と併用する治療戦略を提示した。

このように本論文は、ヒト乳癌細胞に対するAs₂S₂の作用機序を詳細に論じ、また乳癌治
療に新たな選択肢を投じる有用な知見を含んでいることから、博士（薬学）学位論文に値するものと判断される。