Anti-tumor Effect of Arsenic Trioxide (As$_2$O$_3$) on Human Breast Cancer

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Philosophy in Biochemistry

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Abstract

Arsenic Trioxide (As₂O₃) has been explored for its use as medicine in both Western and Chinese societies. It is a major ingredient of arsenic compounds in Traditional Chinese Medicine (TCM) and is derived from Pishi by sublimation. In 1990s, As₂O₃ was reported to treat patients with acute promyelocytic leukemia (APL). Since then, much of study of arsenic therapy has been focused on APL and other leukemia. Only until recently, research on other solid tumor cells was emerged.

In the present study, the mechanism of As₂O₃ treatment of breast cancer, a common cancer in women in Hong Kong and worldwide, was investigated. Two human breast tumor cell lines were used, estrogen receptor alpha (ERα) positive MCF-7 cells line which mimicks early stage of the tumor and ER α negative MDA-MB-231 cells line which mimicks late stage of the tumor. This study finds that As₂O₃ inhibits cell proliferation of MDA-MB-231 cells in a time and dose dependent manner and the IC₅₀ of As₂O₃ of MDA-MB-231 cells were over 20μM, 18.4μM, 12.1μM upon 24, 48, 72 hours treatment respectively. The mechanistic study indicated that As₂O₃ inhibited cell proliferation via cycle arrest and apoptosis. It induced apoptosis via both extrinsic and intrinsic apoptotic pathway by regulating the pro- or anti-apoptotic molecules. Moreover, As₂O₃ induced cell cycle was arrested at G₂ phase in MDA-MB-231 cells.
In *in vivo* study, nude mice bearing MCF-7 cells were also used as animal model to study the potential of As$_2$O$_3$ in breast cancer treatment and the probable side effect. In this study, As$_2$O$_3$ was proved to be effective to inhibit the tumor growth without severe side effects such as liver or heart tissue damage.

The study revealed that As$_2$O$_3$ was more potent than tamoxifen in combating against breast cancers, especially the late stage breast cancers.
摘要

三氧化二砷(砒霜)在中国以及西方社会一直用于治疗皮肤病或溃疡等疾病。三氧化二砷主要从砒石中提取纯化。二十世纪九十年代，三氧化二砷被发现可用于治疗急性早幼粒细胞白血病（APL）。之后，科学家们着手以三氧化二砷治疗其他不同种类的白血病及肿瘤。乳癌在香港和世界都是一种常见肿瘤而传统抗癌药物Tamoxifen对晚期乳癌无效，因此本研究旨在通过研究三氧化二砷对两种不同乳癌细胞株的抑制作用及其作用机制，从而探讨三氧化二砷对乳癌的治疗效用。MCF-7细胞为雌激素生长依赖性，是研究早期乳癌细胞的模型，而MDA-MB-231细胞为雌激素生长非依赖性，为研究晚期乳癌细胞的材料。

体外研究结果显示三氧化二砷能有效抑制乳癌细胞株MDA-MB-231细胞生长。其效力与剂量及时间呈正相关。在用三氧化二砷治疗24, 48, 72小时后，其抑制癌细胞生长百分之五十(IC50)的浓度分别为大于20μM, 18.4μM, 12.1μM。

对其作用机制的研究表明，三氧化二砷通过调节控制细胞凋亡相关蛋白的表达激活不同细胞凋亡路径而诱导细胞程序性死亡，也通过调节cyclin-B蛋白表达阻止G2/M细胞期而抑制MDA-MB-231细胞生长。同时，动物体内研究结果表明，三氧化二砷可在体内有效抑制肿瘤生长不对动物正常肝脏心脏组织造成严重损伤。

总之，与传统抗乳癌药相比，三氧化二砷通过诱导细胞凋亡和阻止细胞周期能更有效治疗乳癌，特别是晚期乳癌。
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>Δψ_m</td>
<td>Mitochondria Transmembrane Potential</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukemia</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>As₂O₃</td>
<td>Arsenic Trioxide</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Transaminase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATRA</td>
<td>All Trans Retinoic Acid</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid Solution</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-Cell Leukemia/Lymphoma-2</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast Cancer Gene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine-containing Aspartate-specific Proteases</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent Kinase</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myeloid Leukemia</td>
</tr>
<tr>
<td>CR</td>
<td>Complete Remission</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal Carcinoma in Situ</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>E-blot buffer</td>
<td>Electroblotting Buffer</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiograms</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-Diamine-Tetra-Acetic Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-2-Hydroxyethyl-1-Piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Inhibitory Concentration</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive Ductal Carcinoma</td>
</tr>
<tr>
<td>ILC</td>
<td>Invasive Lobular Carcinoma</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LD</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activation Protein Kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug Resistance</td>
</tr>
<tr>
<td>MEGM</td>
<td>Mammary Epithelial Cell Growth Medium</td>
</tr>
<tr>
<td>mg</td>
<td>Miligram</td>
</tr>
<tr>
<td>ml</td>
<td>Mililiter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) Polymerase</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral Blood Lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed Cell Death</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic Leukemia Gene</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>RARα</td>
<td>Retinoic Acid Receptor-alpha</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RNase A</td>
<td>Ribonuclease A</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered Saline-Tween-20</td>
</tr>
<tr>
<td>TdP</td>
<td>Torsade de Pointes Tachycardias</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetra-Methylethylenediamine</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by Volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight by Volume</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1 New case numbers of breast cancer by year in Hong Kong 3
Figure 1.2 The chances of getting breast cancer by age 5
Figure 1.3 The incidence and mortality rates of woman with different age getting breast cancer in Hong Kong 6
Figure 1.4 Structures of three estrogen 7
Figure 1.5 The domain structures of estrogen receptor 9
Figure 1.6 The tamoxifen and other related nonsteroidal anti-estrogen 12
Figure 1.7 The appearance of As$_2$O$_3$ and pishi 14
Figure 1.8 The structure of As$_2$O$_3$ 15
Figure 1.9 MCF-7 cells from ATCC 25
Figure 1.10 MDA-MB-231 cells from ATCC 25
Figure 3.1 The anti-proliferate effect on MDA-MB-231 cells studied by MTT assay 49
Figure 3.2 Comparison of anti-proliferate effect of As$_2$O$_3$ and tamoxifen on MDA-MB-231 cells (a) Incubated for 48 hours (b) Incubated for 72 hours 51
Figure 3.3 Study cytotoxicity effects of As$_2$O$_3$ on 184B5 and MDA-MB-231 cells (a) Incubated for 48 hours (b) Incubated for 72 hours 53
Figure 4.1 Cell cycle pattern of MDA-MB-231 cells treated with different concentration of As$_2$O$_3$ for 72 hours 58
Figure 4.2 Result of DNA fragmentation assay of MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours 61
Figure 4.3 The dot plots with the intensity of PI against Annexin V-FITC 63
Figure 4.4 Protein expression level of bcl-2 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours 67
Figure 4.5 Protein expression level of bax in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours 68
Figure 4.6 Protein expression level of cytochrome c in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours 70
Figure 4.7 Protein expression level of caspase 9 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours 72
Figure 4.8 Protein expression level of FasL in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours 74
Figure 4.9  Protein expression level of caspase 8 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours concentration, 72 hour post transfection

Figure 4.10 Protein expression level of caspase 3 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours

Figure 4.11 Protein expression level of PARP in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours

Figure 4.12 Protein expression level of p53 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours

Figure 4.13 Protein expression level of cyclin B1 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours

Figure 4.14 Protein expression level of cyclin E in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours

Figure 5.1 The effect As$_2$O$_3$ on the tumor growth in tumor bearing nude mice during treatment

Figure 5.2 The changes of body weights of tumor bearing nude mice during treatment

Figure 5.3 Effect of As$_2$O$_3$ on Aspartate dehydrogenase (AST) activity of nude mice

Figure 5.4 Effect of As$_2$O$_3$ on Alanine dehydrogenase (ALT) activity of nude mice

Figure 5.5 Effect of As$_2$O$_3$ on Creatin Kinase (CK) activity of nude mice

Figure 5.6 Effect of As$_2$O$_3$ on Lactate dehydrogenase (LDH) activity of nude mice
List of Tables

Table 1.1  Table 1.1 Summary of clinical trials of arsenic trioxide (As$_2$O$_3$) on APL patients  22
Table 3.1  The IC$_{50}$ of As$_2$O$_3$ on MDA-MB-231 cells with 24, 48 and 72 hours treatment by MTT assay  55
Table 3.2  Compare the anti-proliferation effect of As$_2$O$_3$ and Tamoxifen on MDA-MB-231 cells for 48 and 72 hours treatment  55
Table 3.3  Compare the cytotoxicity of As$_2$O$_3$ on 184B5 and MDA-MB-231 cells for 48 and 72 hours treatment  55
Table 4.1  The percentage of MDA-MB-231 cells distributions in sub-G$_1$, G$_1$, S and G$_2$/M phase after treated with different concentrations of As$_2$O$_3$ for 72 hours  59
# Table of Contents

Acknowledgements i  
Abstract ii  
論文摘要 iv  
Abbreviations v  
List of Figures vii  
List of Tables ix  
Table of Contents x

## Chapter 1 Introduction

1.1 Breast Cancer  
1.1.1 Introduction to Breast Cancer 1  
1.1.2 Types of Breast Cancer 3  
1.1.3 Epidemiologic Risk Factors and Etiology 4

1.2 Estrogen and Breast Cancer 7

1.3 Estrogen Receptor 9

1.4 Current Treatment of Breast Cancer 10  
1.4.1 Chemotherapy 10  
1.4.2 Hormonal (Anti-Estrogen) Therapy 11  
1.4.2.1 Tamoxifen and Other Anti-estrogens 12  
1.4.2.2 Disadvantages of Tamoxifen 13

1.5 Arsenic Trioxide 14  
1.5.1 The Characteristics of Arsenic Trioxide (As$_2$O$_3$) 14  
1.5.2 The Medical use of Arsenic Trioxide (As$_2$O$_3$) 16  
1.5.3 Arsenic Trioxide (As$_2$O$_3$) in treating Acute Promyelocytic Leukemia (APL) 17  
1.5.3.1 Acute Promyelocytic Leukemia (APL) 17  
1.5.3.2 All-trans Retinoic Acid (ATRA) Treatment of APL 18  
1.5.3.3 Clinical Trial of the Arsenic Trioxide on APL 19  
1.5.3.4 *In vitro* and *in vivo* Study of Arsenic Trioxide (As$_2$O$_3$) in treating APL 19  
1.5.3.5 Common Side Effects of Arsenic Trioxide (As$_2$O$_3$) on APL 21

1.6 Aim of Study 24
Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Cell Lines and Culture Medium

2.1.1.1 Cell Lines
2.1.1.2 Culture Medium

2.1.2 Chemicals

2.1.3 Buffers and Reagents

2.1.4 Reagents for MTT Assay

2.1.5 Reagents for DNA Fragmentation

2.1.5.1 Reagents for DNA Extraction
2.1.5.2 Reagents for Gel Electrophoresis

2.1.6 Reagents for Western Blotting

2.1.6.1 Reagents for Protein Extraction
2.1.6.2 Reagents for SDS-PAGE

2.1.7 Reagents for Flow Cytometry

2.1.8 In Vivo Study

2.2 Methods

2.2.1 Cell Treatment

2.2.2 Trypan Blue Exclusion Assay

2.2.3 MTT Assay

2.2.4 Detection of DNA Fragmentation

2.2.5 Flow Cytometry

2.2.5.1 Detection of Cell Cycle Pattern with PI
2.2.5.2 Detection of Apoptosis with Annexin V-PI

2.2.6 Western Blot Analysis

2.2.6.1 Protein Extraction
2.2.6.2 Protein Concentration Determination
2.2.6.3 Western Blotting

2.2.7 In Vivo Study

2.2.7.1 Animal Model
2.2.7.2 Treatment Schedule
2.2.7.3 Toxicity of Arsenic Trioxide

Chapter 3 Anti-Proliferation Effect of $\text{As}_2\text{O}_3$ on MDA-MB-231 cells

3.1 Study the Anti-proliferation Effect of $\text{As}_2\text{O}_3$ on MDA-MB-231 Cells by MTT Assay
Chapter 1
Introduction
1.1 Breast Cancer

1.1.1 Introduction to Breast Cancer

Breast cancer, or breast carcinoma, is a cancer in the breast tissue where the proliferation of the cells is out of control and damages the normal breast tissue. It occurs not only in women but also in men.

Breast cancer is the fifth most common cause of cancer death in the world. Among women, breast cancer is the most common cancer and the most common cause of cancer death in the world (World Health Organization, 2007).

An estimated 178,480 new cases of invasive breast cancer are estimated to occur among women in the United States in 2007. An estimated 40,460 women will die from breast cancer. It is forecasted that 2,030 men will be diagnosed and 450 men will die of breast cancer in 2007 in the U.S. (American Cancer Society Cancer Facts & Figures, 2007). Women in the U.S. have 1 in 8 lifetime chance of getting invasive breast cancer and a 1 in 33 chance of breast cancer death (American Cancer Association, 2007). Breast cancer kills more women in the United States than any other cancers except lung cancer.

In Hong Kong, breast cancer is the top cancer among women since 1994. In the year 2001 there were nearly 2000 new cases of breast cancer (Fig. 1.1). One in every 23 Hong Kong women will have breast cancer in their life time. Compared to the Western countries where 1 in every 10 women will have breast cancer, Hong Kong is lower in incidence. However, Hong Kong is above world average in the incidence of breast cancer (Foo, 2007).
1.1.2 Types of Breast Cancer

Breast tumors can be either malignant (cancerous) or benign (non-cancerous). Nine out of ten breast lumps are not cancerous and may be fibroadenomas and cysts. The difference between malignant and benign tumors is that malignant tumors have the ability to invade surrounding areas. Sometimes cells from malignant tumors break away and travel to other areas of the body where they may grow and form 'secondary' breast cancer tumors. Another name for a secondary tumor is a metastasis. The breasts have a rich...
blood supply and an extensive lymph gland drainage system, which contribute factors in the spread of the disease.

Breast cancers are grouped as invasive and non-invasive, depending on their ability to spread to neighboring tissues: The most common type of non-invasive breast cancer is ductal carcinoma in situ (DCIS). Cancer cells are found inside the ducts but have not yet spread through the walls of the ducts into the breast tissue. Most people diagnosed with DCIS at early stage are able to have the cancer completely removed. In some cases, DCIS may develop into an invasive form of breast cancer, if not treated at properly.

The most common type of invasive breast cancer is called invasive ductal carcinoma (IDC) which is responsible for around 80% of all breast cancers. Cancer cells are found both in the ducts and in the breast tissue. These cells are also able to metastasize to other parts of the body.

Invasive lobular carcinoma (ILC) accounts for between 10-15% of all breast cancers. With ILC, cancer cells grow initially in the lobes of the breast and have the ability to spread both to other areas of the breast and to other parts of the body.

1.1.3 Epidemiologic Risk Factors and Etiology

The scientists still do not know exactly why women get breast cancer; however, there are many risk factors that are thought to be related to breast cancer development.

The risk factors that people can not control:
• Age - the chance of getting breast cancer rises as a woman gets older (Fig 1.2 and 1.3)

**Age: a major factor**

A woman's chance of getting breast cancer increases with age. Your chance by your current age is:

<table>
<thead>
<tr>
<th>Age</th>
<th>Chance</th>
</tr>
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<tbody>
<tr>
<td>20</td>
<td>1 in 1,985</td>
</tr>
<tr>
<td>30</td>
<td>1 in 229</td>
</tr>
<tr>
<td>40</td>
<td>1 in 68</td>
</tr>
<tr>
<td>50</td>
<td>1 in 37</td>
</tr>
<tr>
<td>60</td>
<td>1 in 26</td>
</tr>
<tr>
<td>70</td>
<td>1 in 24</td>
</tr>
<tr>
<td>Ever</td>
<td>1 in 8</td>
</tr>
</tbody>
</table>


Fig 1.2 The chances of getting breast cancer by age.
Fig 1.3 The incidence and mortality rates of woman with different age getting breast cancer in Hong Kong

- Genes - there are two genes, BRCA1 and BRCA2 that greatly increase the risk. Women who have family members with breast or ovarian cancer may wish to be tested.

- Personal factors - started menstruating at an early age (before age 12) or went through menopause at a late age (after age 55).

The other risk factors include overweight, using hormone replacement therapy, taking birth control pills, drinking alcohol, not having children or having your first child after age 35 or having dense breasts (National Cancer Institute, NIH, USA).
1.2 Estrogen and Breast Cancer

Estrogen is a group of steroid hormone which is produced primarily in the ovaries. The three major naturally occurring estrogens in women are estradiol, estriol and estrone (Fig 1.4). Breast also produced estrogen in a smaller amount. The secondary source of estrogen is especially important in estrogen production to help control a woman’s menstrual cycle (Purohit et al., 1992).

![](estradiol.png) ![estriol.png] ![estrone.png]

Fig 1.4 Structures of three estrogens

A fundamental theme of more recent studies is that estrogen can modulate a larger repertoire of genes through other mechanisms. These include protein – protein interactions with other DNA binding transcription factors and through the action of membrane associated ER that alters the function of cytoplasmic signal transduction proteins (non-genomic actions of estrogen) (Inoue et al., 2004, Santen et al., 2003).

Estrogens have been found to be the primary stimulant of the breast cancer (Lippman et al., 1976, Umans et al., 1984, Hajek et al., 1997). Reverse effects were shown upon removal of the ovaries and administration of anti-estrogenic drugs or estrogen synthesis.
inhibitors (Nadi et al., 1995). And clinical trials showed that about one-third of women with metastatic breast cancer responded positively to estrogen ablation and hormone therapy (Kyprianou et al., 1991, Fabian et al., 1994).

In vitro, the mechanism study demonstrated that estrogen promoted the cell proliferation and prevented the apoptosis of breast cancer cells. Estrogen require the function of the Ras, mitogen-activated protein kinase (MEK)1 and extracellular signal-regulated kinase (ERK) pathway to induce G1/S phase transition (Ahamed et al., 2001), at elicits down-regulation of the Cdk2 inhibitor p27kip1 (Foster, 2003, Zhu et al., 2003) to increase the cell proliferation by recruit non-cycling cells into cell cycle and to shorten the overall cell cycle time. In the ER-positive MCF-7 breast cancer cell line, anti-apoptotic effects of estrogen have been reported previously (Huang, 1997, Perillo et al., 2000, Ahamed et al., 2001). Studies indicate that estrogen induces transcription of the BCL-2 gene and increases expression of BCL-2 protein, which exerts anti-apoptotic action in many cell types (Huang, 1997, Dong et al., 1999, Leung and Wang, 1999). In addition to genomic actions, estrogen has been known to exert rapid, likely nongenomic actions both in the whole animal and in cultured cells (Szego, 1974, Huang, 1997, Razandi et al., 2000). Similar study has also shown the inhibition of UV radiation, TNF-α, H2O2, or serum withdrawal induced apoptosis by estrogens through the inhibition of JNK activity and BAD activity (Fernando and Wimalasena, 2004).

However, the majority of breast cancers are estrogen independent, that is their development lacks appreciable expression of either the estrogen or progesterone receptors. They are estrogen independent. Recent studies demonstrated that increasing the levels of circulating estrogens is sufficient to promote the formation and progression of estrogen
receptor negative (ER-negative) cancers while, pharmacologically inhibiting estrogen synthesis following pregnancy prevents ER-negative tumor formation. It indicated that estrogen promoted the growth of ER-negative cancers by acting on cells distinct from the cancer cells to stimulate angiogenesis (Gupta and Kuperwasser, 2006).

1.3 Estrogen Receptor

An estrogen receptor (ER) is a nuclear receptor for estrogens such as estradiol. It occurs both on cell membrane and nucleus. Both receptors have functions independent of DNA binding (Levin, 2005). The action of estrogen is mediated through ER.

Recent studies have revealed the existence of two distinct estrogen receptors in our bodies: estrogen receptor alpha (ER\(\alpha\)) and estrogen receptor beta (ER\(\beta\)). While they both bind estrogen as well as other agonists and antagonists, the two receptors have distinctly different localizations and concentrations within our body. Structural differences also exist between the two (Fig 1.5).

![Fig 1.5 The domain structures of estrogen receptor](http://en.wikipedia.org/wiki/Estrogen_receptor)
About 75% of breast cancers are estrogen-receptor-positive ("ER-positive" or "ER+). ERα is certainly associated with more differentiated tumours, while involvement of ERβ remains controversial.

1.4 Current Treatment of Breast Cancer

Today, there's an overwhelming menu of treatment choices for various cancers. The most common steps are as following: surgery first, followed by chemotherapy and/or radiation, and the hormonal (anti-estrogen) therapy is usually started after other treatments have been given.

1.4.1 Chemotherapy

Chemotherapy is the use of drugs to treat cancer. Chemotherapy works best on rapidly dividing cells. Chemotherapy works by stopping the growth or multiplication of cancer cells. Some chemotherapy drugs regulate the estrogen receptors. Estrogens stimulate the growth by regulation of target genes expression with the recruitment of coregulators. Targeting at these coregulators can alter the estrogens regulated gene expression and in turn the growth of breast cancer (Bevan and Park, 1999).

In recent years, induction of apoptosis becomes another important mechanism in the cancer chemotherapy. The studies show that cytotoxic chemotherapy induced increase in apoptosis during treatment (Dive et al., 1992). Further molecular studies demonstrated that this "dormant" population was important to characterize the mechanism of their resistance to drug therapy (Dowsett et al., 1999).
1.4.2 Hormonal (Anti-Estrogenic) Therapy

As the estrogens and estrogen receptors involve in breast cancer development, hormonal therapy can be a very important part of breast cancer treatment. It is especially against breast cancer that is hormone-receptor-positive. There are four major kinds of hormonal therapy. Depending on personal situation of the patients, doctor will decide which one is most appreciate for you. Some of the treatments lower the amount of estrogen in the body (Aromatase inhibitors), some block estrogen’s ability to lock onto the estrogen receptor (selective estrogen-receptor modulators), some destroy the estrogen receptor (estrogen-receptor downregulators) and some remove or shut down the major source of estrogen production (ovarian shutdown or removal) (breastcancer.org).

1.4.2.1 Tamoxifen and Other Anti-estrogens

The anti-estrogen drug that has been used most often is tamoxifen. Tamoxifen is also used to treat metastatic breast cancer and to prevent the development of breast cancer in a woman at high risk. Tamoxifen worked as an anti-estrogen. It was able to treat human breast and on rat mammary tumor and prevent the induction and growth of ER positive carcinogen-induced rat mammary carcinomas (Jordan et al., 1976).
Fig 1.6 The tamoxifen and other related nonsteroidal anti-estrogen (Jordana and Brodieb, 2007).

The translational research with tamoxifen to target the ER with the appropriate duration (5 years) of adjuvant therapy has contributed to the falling national death rates from breast cancer in the U.S. (National Cancer Institute, NCI). Additionally, exploration of the endocrine pharmacology of tamoxifen and related non-steroidal anti-estrogen (e.g. keoxifene now known as raloxifene) resulted in the recognition of selective ER modulation and the translation of the concept to use raloxifene for the prevention of osteoporosis and breast cancer (Jordana and Brodieb, 2007) (Fig 1.6).
1.4.2.2 Disadvantages of Tamoxifen

Firstly, tamoxifen is the hormonal treatment as choice for pre-menopausal women and shows ineffective in estrogen-receptor (ER) negative patients (i.e. advanced breast cancer patients). It has been reported that tamoxifen had little or no effect on estrogen-receptor-negative tumor cells in culture or on tumors in animals (Lippman et al., 1977, Osborne et al., 1985). Furthermore, responses are uncommon (5 to 10 percent) in women with estrogen-receptor-negative metastatic breast cancer treated with the drug (Osborne et al., 1980). A 1992 meta-analysis of trials of adjuvant therapy suggested that tamoxifen had a small but statistically significant survival benefit in these women (Early Breast Cancer Trialists Collaborative Group, 1992), but the updated meta-analysis with longer follow-up and additional women did not support the conclusion (Early Breast Cancer Trialists Collaborative Group, 1998).

Secondly, beside common side effects caused by all treatments of breast cancer, tamoxifen may cause some severe side effects, including blood clots (thrombosis), endometrial cancer (cancer of the uterine lining), abnormal growth of uterine tissue (endometriosis), stroke, and fertility issues (Osborne, 1998, Clemons et al., 2002).

Finally, patients may develop drug resistance within 2-5 years treatment. Over time, tamoxifen may lose its anti-estrogen powers. It may end up having no effect that can be measured or seen on the breast cells, or even start working like a real estrogen, and begin stimulating cancer cell growth instead of decreasing it (Osborne, 1998, Riggins et al., 2007). Two forms of anti-estrogen resistance occur: de novo resistance and acquired resistance. Absence of estrogen receptor (ER) expression is the most common de novo resistance mechanism, whereas a complete loss of ER expression is not common in
acquired resistance. Anti-estrogen unresponsiveness appears to be the major acquired resistance phenotype, with a switch to an anti-estrogen-stimulated growth being a minor phenotype. A loss of anti-estrogen responsiveness by initially responsive tumors is likely to be the most common acquired resistance phenotype. A different resistance phenotype has been described in human breast cancer xenografts that exhibit a switch to a tamoxifen (TAM)-stimulated phenotype (Clemons et al., 2002, Clarke et al., 2003).

1.5 Arsenic Trioxide (As$_2$O$_3$)

1.5.1 The Characteristics of Arsenic Trioxide (As$_2$O$_3$)

Arsenic trioxide (As$_2$O$_3$) is the most important commercial compound of arsenic, and the main starting material for arsenic chemistry. It appears as odorless, tasteless and white crystals or powders (Fig 1.7). The structure of As$_2$O$_3$ is a ring shape with formula As$_4$O$_6$ (Gorby, 1994) (Fig 1.8). In traditional Chinese medicine, it is called pishuang (砒霜). It can be prepared by purifying from a naturally occurring complex called pishi (砒石) which composed 90% of As$_2$O$_3$ (Fig 1.7) (中医药发展筹备委员会，1997).
Arsenic is ubiquitous in the natural environment. Arsenic trioxide is one of the arsenites, the inorganic forms of arsenic in the trivalent state $\text{As}^{3+}$, found in nature and is produced mainly from products of copper smelting.

In the past, Arsenic is considered to be toxic drugs, so its primarily application is used for wood preservatives, insecticides, and herbicides. It is also used in metallurgical processes, and in the manufacturing of glass and ceramics.

Arsenic compounds were formerly used medicinally in humans in both Western and Chinese societies in treating some severe diseases. In Western medicine, $\text{As}_2\text{O}_3$ was prepared to the Fowler’s solution to treat hemorrhoids, acute ulcerative gingivitis and asthma externally. And in Chinese society, $\text{As}_2\text{O}_3$ also has been used for many decades in traditional Chinese medicines for treatment of various human diseases like ulcer and
1.5.2 The Medical use of Arsenic Trioxide (As₂O₃)

Arsenic has been used as a therapeutic agent for more than 2400 years in different parts of the world. In 15th century, discovered by William Withering, As₂O₃ was used to treat digitalis (Aronson, 1994). In 18th century, Thomas Fowler compounded a potassium bicarbonate-based solution of As₂O₃ named Fowler's Solution for the treatment of a variety of disease until early 20th century. As early as in 19th century Fowler's solution was used as a treatment of choice for chronic myeloid leukemia (CML). However, due to toxic side effects of long-term heroic-dose of oral arsenic in most patients, and with the advent of modern radiotherapy and chemotherapy, the arsenic treatment for CML was given up in Western medicine (Kwong and Todd, 1997, Tamm et al., 1998).

As in traditional Chinese medicine, the medical application of As₂O₃ was found in the Compendium of Materia Medica by Mr Li Shi-Zhen 李时珍 (1518 - 1593) (Zhang et al., 2001). It was used to treat skin diseases and asthma (Wang et al., 2001). However, As₂O₃ was considered as a poison for a long time. In early 1970s, a discovery from China revived this drug. A group of researchers from Harbin Medical University in Northeast China found that intravenous administration of arsenic trioxide (As₂O₃) with relatively small doses (10 mg/d) was effective in treating patients with APL, lymphoma, and liver cancer (Zhang, 1988, Li et al., 1988, Sun et al., 1992). Then, in 1992, Sun et al reported promising results of 32 cases of APL treated with As₂O₃ (Sun et al., 1992). Later, a trial performed at the Shanghai Second Medical University indicated that complete remission (CR) could be achieved in 14 of 15 investigated patients that had relapsed after prior
treatment with all-trans retinoic acid (ATRA) or conventional chemotherapy (Shen et al., 1997). In the Western, the population clinical efficacy of As$_2$O$_3$ was proved by studies performed by Soignet et al (Soignet et al., 1998, Soignet et al., 1999). Recently, the drug (Trisenox$^\text{TM}$) was formally approved by the State Food and Drug Administration (SFDA) in China (1999) and the Food and Drug Administration (FDA) of the United States of America (2000).

1.5.3 Arsenic Trioxide (As$_2$O$_3$) in treating Acute Promyelocytic Leukemia (APL)

1.5.3.1 Acute Promyelocytic Leukemia (APL)

Acute promyelocytic leukemia (APL or AML-M3 and M3-variant) represents a unique model for cancer research in terms of the biological and clinical features. It has been shown during the last decade that PML-RARa chimeric protein, as a result of the specific chromosome translocation t(15; 17), plays a central role in APL pathogenesis (Chen et al., 1997). APL is characterized by chromosomal translocation involving the retinoic acid receptor-alpha gene on chromosome 17 (RARa). In 95% of APL cases, retinoic acid receptor-alpha (RARa) gene on chromosome 17 is involved in a reciprocal translocation with the promyelocytic leukemia gene (PML) on chromosome 15, a translocation denoted as t(15;17)(q22;q12). Four other gene rearrangements including fusing RARa to promyelocytic leukemia zinc finger (PLZF), nucleophosmin (NPM), nuclear matrix associated (NUMA), or signal transducer and activator of transcription 5b (STAT5B) genes have been described in APL. The resultant fusion proteins disrupt the
function of RARα which blocks the normal maturation of granulocytes. Although the chromosomal translocation involving "RARα" is believed to be the initiating event, additional mutations are required for the development of leukemia (Kumar et al., 2004).

1.5.3.2 All-trans Retinoic Acid (ATRA) Treatment of APL

Before the 1990s, patients with APL were treated with anthracyclines combined with cytosine arabinoside (Bernard et al., 1973). In the First reports of ATRA therapy published by the research groups in Shanghai and other countries, CR rates of about 90% were reported in newly diagnosed and first relapse APL patients treated with a dose of 45 mg/m² daily ATRA (Huang et al., 1988, Castaigne et al., 1990, Degos et al., 1990, Chen et al., 1991, Warrel et al., 1991). The in vitro and in vivo studies indicated that ATRA induced a terminal differentiation followed by a natural apoptosis of malignant cells and is, thus, the leading drug for modifying the biological process of malignant cells, so it was effective essentially in the leukemia-bearing specific chromosome aberration t(15;17) with PML/RARα fusion gene. The treatment of APL with ATRA constitutes a successful model for gene-targeting therapy (Degos and Wang, 2001).

Although the ATRA is found to cause an increase in CR rate, some adverse effects also found during or after treatment. In 30% of the patients treated with ATRA as a single drug was associated with rapid increase in leukocytes and signs of "ATRA syndrome", which could have fatal outcome (De Botton et al., 1998). And following treatment with ATRA alone, patients experienced an induction of a secondary resistance to ATRA. Secondary resistance occurs in all patients treated with ATRA (Warrell et al., 1994). Thus, patients receiving ATRA alone achieved CR for only a short time, usually
relapsing within 6 months. Patients treated with ATRA become refractory to this treatment for a period of 6 - 12 months and the acquired resistance is usually reversible after this period (Degos and Wang, 2001). These side effects promote the scientists to modify the treatment of APL.

1.5.3.3 Clinical Trial of the Arsenic Trioxide on APL

Since the ATRA has the adverse effects mentioned above, scientists started to modify the treatment. Based on the principle in Chinese traditional medicine of "use a toxic agent against a toxic agent," in the early 1970s a group of clinical researchers at Harbin Medical University began to treat some types of cancer with white arsenic, which is known to be a toxin. Encouraging results have been observed in the treatment of esophageal carcinoma, malignant lymphoma, and leukemia, particularly CML and acute promyelocytic leukemia (APL) (Wang, 2001). Then clinical trials were done by research groups in Chinese and western countries in the following years. (Shown in Table 1.1) Generally arsenic trioxide achieved complete remission (CR) rate of 57-98% in APL patients. (Zhang et al., 2001)

1.5.3.4 In vitro and in vivo Study of Arsenic Trioxide ($\text{As}_2\text{O}_3$) in treating APL

After 1970s when $\text{As}_2\text{O}_3$ were found to be effect in treating APL patients, the action mechanism studies of $\text{As}_2\text{O}_3$ were followed. NB4 cells, an APL cell line with chromosome translocation t(15:17) from a relapsed APL patient were used in the in vitro study. $\text{As}_2\text{O}_3$ was found to have a dose-dependent dual effect: inducing preferentially
apoptosis at relatively high concentrations (0.5 to 2 mmol/L) and inducing partial, differentiation at low concentrations (0.1 to 0.5 mmol/L) (Chen et al., 1997). On one hand, in high concentrations, As$_2$O$_3$ induced apoptosis in APL. The most important mechanism is degradation of the APL-specific PML/RAR$\alpha$ fusion transcript. As$_2$O$_3$ induced a significant modulation of the PML staining pattern in NB4 cells and HL-60 cells (Chen et al., 1996, Shao et al., 1998). The characteristics of PML-RAR$\alpha$ in NB cells disappear after treatment with As$_2$O$_3$, whereas a diffuse PML staining occurs in the perinuclear cytoplasmic region. Other mechanism reported include induction of apoptosis through modulate effects on pro-and anti-apoptotic molecules such as bax, bcl-2 gene expression at both mRNA and protein levels (Chen et al., 1996, Kinjo et al, 2000). In addition, As$_2$O$_3$ induced apoptosis by enhancing the reactive oxygen species (ROS) content and the activity of caspase 3 in NB4 cells, thus damaging the mitochondrial membrane (Wang, 2001). Further study also indicated that As$_2$O$_3$ induces apoptosis without differentiation in retinoic acid-sensitive and retinoic acid resistant APL cells at concentrations that are achievable in patients (Shao et al., 1998, Kinjo et al, 2000).

On the other hand, in vitro and in vivo experiments have demonstrated that low-dose As$_2$O$_3$ induces differentiation of NB4 cells as well as fresh APL cells. The differentiation is characterized by maturation of morphology, elevation of cytoplasmic-nuclear ratio. Condensation of chromatin structure, appearance of neutrophilic granules, increased expression of CD11b and adhesion molecules and decreased expression of CD33 (Chen et al., 2001). As$_2$O$_3$ could induce degradation of both PML-RAR$\alpha$ fusion protein and wild-type PML protein in APL cells. These, together with the fact that As$_2$O$_3$ could not induce the degradation of PLZF-RAR$\alpha$, suggest PML moiety is the target of As$_2$O$_3$. 

20
As$_2$O$_3$-induced degradation was also associated with the distribution of PML proteins to nuclear matrix and the conjugation with a ubiquitin-related protein SUMO-1 (Sternsdorf et al., 1999). Further investigation showed the upregulation of CD52 and BFI-1, downregulation of RAR$\alpha$ and synergistic effects in regulation of protein kinase CB-1 (Cai et al., 2000). Recent findings showed that arsenic trioxide induces acetylation of histones 3, 4 resulting in transcriptional activation of downstream genes for differentiation (Fang et al., 2002).

1.5.3.5 Common Side Effects of Arsenic Trioxide (As$_2$O$_3$) on APL

Of course, known as a poison for many centuries, As$_2$O$_3$ causes multiple adverse effects during treating APL, including hyperglycemia, dyspnea, QT prolongation, torsade de pointes tachycardias (TdP), sudden cardiac death, fatal APL differentiating syndrome (characterized by fever, dyspnea, weight gain, pulmonary infiltrates and pleural or pericardial effusions, with or without leukocytosis) (St. Petery et al., 1970; Shen et al., 1997, Ohnishi et al., 2000; Unnikrishnan et al., 2001; Westervelt et al., 2001). However, treatment with As$_2$O$_3$ was not associated with significant BM suppression-no significant changes of hemoglobin (Hb) and platelet levels were observed in patients. Arsenic trioxide treatment does not cause hair loss, mouth sores and doesn't significantly lower blood counts like other chemotherapy. Moreover, although about 40% percent of patients experienced a QT prolongation in As$_2$O$_3$ treatment with APL, almost all of them recovered after the treatment were stopped. The patients' electrocardiograms (ECG) need to be closely monitored during treatment. Therefore, according to the report by Shen et al in 1997, Clinical observations revealed that the currently used dose (10 mg) of As$_2$O$_3$
through administration resulted in only mild to moderate side effects (Shen et al., 1997).

Table 1.1 Summary of clinical trials of arsenic trioxide (As$_2$O$_3$) on APL patients (Wang, 2001)

<table>
<thead>
<tr>
<th>Year</th>
<th>Compound</th>
<th>No. of patients</th>
<th>CR rate (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>Ailing-1</td>
<td>32</td>
<td>85.5</td>
<td>Sun et al., 1992</td>
</tr>
<tr>
<td>1995</td>
<td>Composite Indigo</td>
<td>60</td>
<td>98</td>
<td>Huang et al., 1998</td>
</tr>
<tr>
<td>1996</td>
<td>As$_2$O$_3$</td>
<td>30</td>
<td>73.3</td>
<td>Zhang et al., 1996</td>
</tr>
<tr>
<td>1996</td>
<td>As$_2$O$_3$</td>
<td>42</td>
<td>52.4</td>
<td>Zhang et al., 1996</td>
</tr>
<tr>
<td>1997</td>
<td>Ailing-1</td>
<td>15</td>
<td>93</td>
<td>Shen et al., 1997</td>
</tr>
<tr>
<td>1998</td>
<td>As$_2$O$_3$</td>
<td>12</td>
<td>92</td>
<td>Soignet et al., 1998</td>
</tr>
<tr>
<td>1998</td>
<td>As$_2$O$_3$</td>
<td>5</td>
<td>57</td>
<td>Huang et al., 1998</td>
</tr>
<tr>
<td>1998</td>
<td>As$_2$O$_3$</td>
<td>98</td>
<td>87.1</td>
<td>Ma et al., 1998</td>
</tr>
<tr>
<td>1999</td>
<td>As$_2$O$_3$</td>
<td>11</td>
<td>72.7</td>
<td>Niu et al., 1999</td>
</tr>
<tr>
<td>1999</td>
<td>As$_2$O$_3$</td>
<td>47</td>
<td>85.1</td>
<td>Niu et al., 1999</td>
</tr>
<tr>
<td>1999</td>
<td>As$_2$O$_3$</td>
<td>40</td>
<td>85</td>
<td>Soignet et al., 1999</td>
</tr>
<tr>
<td>1999</td>
<td>As$_4$S$_4$</td>
<td>100</td>
<td>84.9</td>
<td>Lu et al., 1999</td>
</tr>
<tr>
<td>2000</td>
<td>As$_2$O$_3$</td>
<td>124</td>
<td>87.9</td>
<td>Zhang et al., 2000</td>
</tr>
<tr>
<td>2000</td>
<td>As$_2$O$_3$</td>
<td>242</td>
<td>74.8</td>
<td>Zhang et al., 2000</td>
</tr>
</tbody>
</table>
1.5.4 Anti-cancer effect of Arsenic Trioxide on other cancers

Apart from APL, As$_2$O$_3$ inhibits growth and promotes apoptosis in other hematological cancer cell lines.

Zhang et al. have reported that at a concentration of 1 μM, As$_2$O$_3$ markedly inhibits both proliferation and viability of NB4 cells, myeloma NOP-1 cells, lymphoma NOL-3 cells, myeloid NKM-1 cells, normal peripheral blood lymphocytes (PBLs), non-Hodgkin's lymphoma (NHL) cells, and chronic lymphocytic leukemia (CLL) cells (Zhang et al., 1998). In 1999, Studies performed by Zhu et al. have revealed that As$_2$O$_3$ markedly inhibits growth in a dose- and time-dependent manner in eight lymphoproliferative disease cell lines: Nalm-6; Namalwa and Raji; BJAB; Su-DHL-4; Molt-4 Jurkat; and SKW-3. It induced apoptosis only in Nalm-6, Namalwa, Raji, Su-DHL-4, and Jurkat cells. The apoptosis induced by As$_2$O$_3$ is associated with disruption of mitochondria transmembrane potential ($\Delta\psi_m$) and caspase 3 activation. Degradation of PML protein also occurred, and there were no alterations in c-myc, Rb, CDK4, cyclin D1, p16, and p53 (Zhu et al., 1999). Arsenic trioxide also inhibited proliferation in K562 cells by inducing G2/M phase cell cycle and upregulating Survivin mRNA expression and content of Survivin protein (Wu et al., 2004).

As$_2$O$_3$ also has anti-proliferation effect and induce apoptosis on solid tumors such as prostate cancer, renal cancer, cervical cancer, hepatoma, breast cancer and so on. Lin C et al. reported As$_2$O$_3$ induced G2+M arrest of tumor cells such as GLC-82, MGC-803, SGC-7901, Eca109 and HeLa cells before induced apoptosis and decreased c-myc gene expression, whereas caused G1 block of HPV16 DNA immortalized cervical epithelial
HCE16/3 cells and did not affect c-myc expression (Lin et al., 2000). Park et al., reported Arsenic trioxide inhibited the proliferation of renal cell carcinoma cell lines (ACHN, A498, Caki-2, Cos-7, and Renca) by inducing a G1 or a G2-M phase arrest in these cells. And it decreased the levels of CDK2, CDK 6, cyclin D1, cyclin E, and cyclin A proteins in A498 cells. And apoptotic process of A498 cells was associate with Bcl-xl, caspase 9, caspase 3, and caspase 7 proteins as with as Δψm loss (Park et al., 2003). Shao et al reported that As2O3 induced dose-dependent apoptosis in gastric carcinoma cells, MKN45 cells, blocked at G2/M phase (Shao et al., 2005). Chan et al reported As2O3 was effective in inhibiting the cell proliferation of R-HepG2 cells in a dose- and time-dependent manner via induction of apoptosis without affecting the cells cycle (Chan et al., 2006). And Li X et al reported that arsenic trioxide cause redistribution of cell cycle, caspase activation, and GADD expression in human colonic (HT-29), breast (MCF-7) and Pancreatic (AsPC-1) cancer cells (Li et al., 2004).

### 1.6 Aim of the Study

This study investigates the effects of As2O3 on human breast tumor cell lines, MCF-7 (Fig 1.9) and MDA-MB-231 (Fig 1.10). MCF-7 cells are estrogen receptor (ER) α positive whereas MDA-MB-231 cells are estrogen receptor ERα negative, mimicking the two classes of breast cancer.

The aim of this study is to (1) explore the potential anti-cancer effect of As2O3 on MDA-MB-231 cells; (2) the action mechanisms of As2O3 on MDA-MB-231 cells (3) in vivo study of anti-tumor effect of As2O3 in breast cancer.
Fig 1.9 MCF-7 cells from ATCC

Fig 1.10 MDA-MB-231 cells from ATCC
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Cell Lines and Culture Medium

2.1.1.1 Cell Lines

MCF-7 and MDA-MB-231 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Both cell lines were maintained in complete RPMI 1640 medium (Invitrogen, USA), which was supplemented with 10% fetal bovine serum (v/v, FBS, Invitrogen, USA) and 1% penicillin-streptomycin (v/v, PS, 10 000U/ml, Invitrogen, USA). The cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide (CO₂). Cells were cultured in 150cm² culture flask (IWAKI) and were sub-cultured twice a week. 184B5 cell was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in complete mammary epithelial cell growth medium (MEGM; Clonetics) supplemented with MEGM bullet kit (Clonetics), which contains EGF, insulin, hydrocortisone, bovine pituitary extract, 10 ng/mL hEGF, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 50 μg/mL gentamicine, and 52 μg/mL bovine pituitary extract at 37 °C in a humidified atmosphere of 5% CO₂. The cells were cultured in 150cm² and were sub-cultured twice a week.

2.1.1.2 Culture Media

Roswell Park Memorial Institute Tissue Culture Medium 1640 (RPMI 1640 Medium, Introgen, USA)

RPMI 1640 medium was prepared by dissolving the powder containing phenol red, L-glutamine and 0.5mM HEPES in 1L of dH₂O. The medium was supplemented with 2g of NaHCO₃ every 5L. The pH was adjusted to 7.2. The medium was then filtered by 0.22μm
bottle-top filter (Millipore). The complete RPMI 1640 medium was supplemented with 10% fetal bovine serum (v/v, FBS, Invitrogen, USA) and 1% penicillin-streptomycin (v/v, PS, 10000U/ml, Invitrogen, USA). The media were stored at 4 °C.

Mammary Epithelial Cell Growth Medium (MEGM; Clonetics CA, USA)
The complete MEGM medium was supplemented EGF, insulin, hydrocortisone, bovine pituitary extract. 10ng/mL hEGF, 5μg/mL insulin, 0.5μg/mL hydrocortisone, 50μg/mL gentamicine, and 52μg/mL bovine pituitary extract. The media were stored at 4 °C.

2.1.2 Chemicals

Arsenic Trioxide ($\text{As}_2\text{O}_3$) was purchased from Sigma Chemical Company. It was prepared by dissolving powder $\text{As}_2\text{O}_3$ in boiled phosphate buffered saline (PBS) in 10mM. Boiling was continued until $\text{As}_2\text{O}_3$ powder was completely dissolved. Distilled water was added to compensate the loss of water vapor during boiling. The stock solution was sterilized by using 0.22μM filter (Millipore) and stored at -20 °C. Aliquots of 10mM were prepared for in vitro studies, which was further diluted to working concentration with culture medium.

Tamoxifen was purchased from Sigma Chemical Company. Stock solution of 10 μM was freshly prepared by dissolving in absolute ethanol and sterilized with 0.22μM filter (Millipore). Working solutions with various concentrations was prepared with culture medium for in vitro culture.
17-estradiol 60-day Release Pellets were purchase form Innovative Research of America. Each pellet contains 0.72mg 17-estradiol with biodegradable carrier-binder. The pellets were stored at room temperature for in vivo study use.

2.1.3 Buffers and Reagents

Trypsin-EDTA Solution

Trypsin-EDTA solution, containing 0.25% Trypsin and 1 mM EDTA-tetrasodium in HBSS without Ca\(^{2+}\) and Mg\(^{2+}\), was purchased from Invitrogen, USA.

Trypan Blue Solution

Trypan blue solution was purchased from Sigma Chemical Co. It contained 0.4% (w/v) trypan blue dissolved in 0.817% (w/v) NaCl and 0.06% (w/v) K\(_2\)PO\(_4\).

10x Tris-Glycine

10x Tris-glycine was prepared by dissolving 30.3g Tris and 144g glycine in 1L dH\(_2\)O. The pH was adjusted to 8.3 and was stored at 4°C.

Normal Saline: 0.9% (w/v) Sodium Chloride (NaCl)

0.9% NaCl was prepared by dissolving 9g of solid form of NaCl in 1L of dH\(_2\)O at room temperature.
Phosphate Buffered Saline (PBS)

PBS was prepared by mixing 136mM NaCl, 2.7mM KCl, 1.5mM KH₂PO₄ and 8mM Na₂PO₄. The chemicals are dissolved in ddH₂O. The pH was adjusted to 7.4, the solution was sterilized by autoclaving, stored at room temperature.

Tris Buffered Saline (TBS)

TBS was prepared by dissolving 12.114g Tris and 87.66g NaCl in 1L dH₂O. The pH was adjusted to 8 and stored at 4°C.

Tris-acetate (TAE) Buffer

TAE was prepared as 50x concentrated stock solution by dissolving 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0) in 1L dH₂O.

Tris-EDTA (TE) Buffer

TE was prepared by mixing 10mM Tris-Cl and 1mM EDTA in dH₂O. The pH was adjusted to 7.4.

2.1.4 Reagents for MTT Assay

Tetrazolium Salt 3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT, Sigma) Solution

MTT was prepared by dissolving it in PBS at concentration of 5mg/ml. The solution was then filtered by a 0.22μm filter (Millipore) and stored at 4°C in dark.
2.1.5 Reagents for DNA Fragmentation Assay

2.1.5.1 Reagents for DNA Extraction

DNA Lysis Buffer

Lysis buffer was prepared by mixing 200mM Tris-HCL buffer (pH 8.3), 100mM EDTA and 1% SDS (w/v) in PBS. It was stored at room temperature.

Proteinase K

Proteinase K was purchased from Sigma chemical Co. It was dissolved in PBS. It was stored at -20 °C.

Ribonuclease A (RNase A)

Ribonuclease A (RNase A) was purchased from Sigma Chemical Co. It was dissolved in TE buffer (5mg/ml, pH 7.4) and stored at -20°C.

2.1.5.2 Reagents for Gel Electrophoresis

1.5% Agarose Gel (w/v)

1.5% agarose gel was prepared by dissolving 0.75 agarose (Sigma Chemical Co.) in 50ml TAE. It was boiled until the agarose was melted. The gel was cooled down to about 50°C and 5μl of ethidium bromide (EtBr) (Amersham Biosciences Ltd.) was added. It was then poured into a cast gel system.
6x DNA Loading Dye

6x DNA loading dye was prepared by mixing 0.25% (v/v) bromophenol blue, 0.25% (v/v) xylene cyanol FF and 30% (v/v) glycerol in dH₂O.

DNA Marker

100 Base-Pair DNA Marker was prepared by mixing 3µl of 1µg/µl of the 100 base-pair DNA marker (Amersham Biosciences Ltd.), 4µl of 6x DNA loading dye and 5µl autoclaved dH₂O.

2.1.6 Reagents and Chemicals for Western Blotting

2.1.6.1 Reagents for Protein Extraction

Protein Lysis Buffer

Lysis buffer was prepared by mixing 21µg/ml aprotinin, 5µg/ml leupeptin, 5mM MgCl₂, 1mM phenylmethylsulfonylfluoride (PMSF), 1% (w/v) SDS, 1mM sodium metavananate (Na₃VO₄) and 10mM Tris buffer (pH 7.4) in PBS, stored at room temperature.

Protein Standard (2mg/ml)

Protein standard was prepared by dissolving 0.004g bovine serum albumin (BSA) in 2ml dH₂O. It was stored at -20°C.
**Bicinchoninic acid (BCA) solution (Reagent A)**

BCA solution was purchased from Sigma Chemical Co. and was stored at room temperature.

**Reagent B**

Reagent B was prepared by dissolving 4g of CuSO₄·5H₂O in 100ml dH₂O. It was stored at room temperature. Mixture of BCA solution and reagent B should be freshly prepared each time.

### 2.1.6.2 Reagents for SDS-PAGE

**10% Ammonium Persulfate (APS)**

APS was prepared by dissolving 1g ammonium persulfate powder in 10ml dH₂O. It was stored at -20°C.

**4x Lower Gel Buffer**

4x lower gel buffer was prepared by dissolving 181.6g Tris and 4g SDS in 1L of dH₂O. The pH was adjusted to 8.8 and it was stored at room temperature.

**4x Upper Gel Buffer**

4x upper gel buffer was prepared by dissolving 60.6g Tris and 4g SDS in 1L of dH₂O. The pH was adjusted to 8.8 and it was stored at room temperature.
**12.5% Separating Gel for SDS-PAGE**

12.5% separating gel was prepared by mixing 1.0825 ml of dH₂O, 1.6675 ml of 30% acrylamide, 1ml of 4X upper gel buffer, 4.65µl of N, N, N', N'-tetramethyl-ethylene diamine (TEMED) and 20µl of 10% APS. Mixture should be freshly prepared for each experiment.

**4.5% Stacking Gel for SDS-PAGE**

4.5% stacking gel was prepared by mixing 1.2ml of dH₂O, 0.3ml of 30% acrylamide, 0.5ml 4X lower gel buffer, 2.65µl of TEMED and 15µl of 10% APS. Mixture should be freshly prepared for each experiment.

**2x SDS Loading Dye**

2x SDS loading dye was prepared by dissolving 2% (w/v) SDS, 10% sucrose, 0.002% bromophenol blue and 62.5mM Tris in dH₂O. The pH was adjusted to 6.8. Then the loading buffer was supplemented with 5% (v/v) 2-mercaptoethanol and stored at 4°C.

**10x SDS Running Buffer**

10x SDS running buffer was prepared by dissolving 30.3g Tris, 144g glycine and 10g SDS in 1L of dH₂O.

**Rainbow™ Coloured Protein Molecular Weight Marker**
Rainbow™ coloured protein molecular weight marker was purchased from Amersham Biosciences Ltd.

**Polyvinylidene Difluoride (PVDF) Western Blotting Membrane**

PVDF membrane with a pore size of 0.45 μm was purchased from Millipore Corporation.

**Electroblotting Buffer**

Electroblotting buffer was prepared by mixing 66.7ml of 10x Tris-glycine, 100ml methanol and 500ml of dH2O.

**Tris-Buffered Saline with 0.1% Tween-20 (TBS-T)**

Tris-Buffered Saline with 0.1% Tween-20 (TBST) was prepared by mixing 1ml of Tween-20 in 1L TBS.

**Blocking Solution**

Blocking solution was prepared by dissolving 1g of non-fat milk powder in 10ml of TBST. It was stored at 4°C.

**Primary and Secondary Antibodies**

The primary antibodies included mouse monoclonal anti-human β-actin (Sigma), mouse monoclonal anti-human bcl-2(Santa Cruz Biotechnology Inc), mouse monoclonal anti-human bax (Santa Cruz Biotechnology Inc), mouse monoclonal anti-human caspase 9 (Stressgen), rabbit polyclonal anti-human cytochrome c (Santa Cruz Biotechnology Inc), rabbit polyclonal anti-human FasL (Santa Cruz Biotechnology Inc), mouse monoclonal
anti-human caspase 8 (BD Biosciences), mouse monoclonal anti-human caspase 3 (Santa Cruz Biotechnology Inc), rabbit polyclonal anti-human PARP (Santa Cruz Biotechnology Inc), mouse monoclonal anti-human p53 (Santa Cruz Biotechnology Inc), Rabbit monoclonal anti-human cyclin B1 (Abcam) and mouse monoclonal anti-human cyclin E (BD Biosciences). They were stored at -20°C. The secondary antibodies included goat anti-mouse IgG and goat anti-rabbit IgG HRP were purchased from Pharmigen and Santa Cruz Biotechnology Inc. respectively. They were stored at 4°C.

**Enhanced Chemiluminescence (ECL) Assay**

Enhanced chemiluminescence (ECL) assay kit was purchased from Amersham Biosciences Ltd. It was stored at 4°C.

**2.1.7 Reagents for Flow Cytometry**

**BD Pharmingen™ Annexin V Kits (Bio-Gene Technology Ltd.)**

BD Pharmingen™ Kits was purchased from Bio-Gene Technology Ltd. The kit contains Annexin V-FITC, 10×Binding Buffer and Propidium Iodide. It was stored at 4°C.

**Propidium Iodide (PI)**

Propidium iodide was purchased from Sigma Chemical Co. It was reconstituted in PBS (2mg/ml).

**Ribonuclease A (RNase A)**
Ribonuclease A (RNase A) was purchased from Sigma Chemical Co. It was dissolved in TE buffer (5mg/ml, pH 7.4).

2.1.8 *In Vivo* Study

**Animal Model: Balb/c Nude Mice**

Female Balb/c nude mice aged 4-6 weeks were used as animal models for *in vivo* study. The mice were bred at the Laboratory Animal Services Center of The Chinese University of Hong Kong under pathogen-free condition in air-controlled rooms.

**Sodium Pentobarbital Solution**

Sodium pentobarbital solution was prepared by dissolving 25mg of sodium pentobarbital in 1 ml of sterile PBS. It was stored at 4°C.

**Heparin Solution**

Heparin solution was prepared by dissolving 50 units of heparin in 1 ml of sterile PBS. It was stored at 4°C.
2.2 Methods

2.2.1 Cell Treatment

Cells were incubated with RPMI 1640 or MEGM medium for 24 hours at 37°C, 5% CO₂ and then the medium was discarded and As₂O₃ was added with fresh medium for 24, 48 and 72 hours.

2.2.2 Trypan Blue Exclusion Assay

0.4% trypan blue solution was mixed with 10μl of cell suspension. Then, 10μl of the solution was transferred to the hemacytometer. The viable cells, which were un-stained, were counted in a square of hemacytometer. The cell concentration (cells/ml) was calculated as follows:

\[
\text{Cells per ml} = \text{number of cell} \times 10^4 \times \text{dilution factor}
\]

2.2.3 MTT assay

1×10⁴ cells per well were seeded onto a 96-well plate in 100μl RMPI 1640 or MEGM medium at 37°C, 5% CO₂. After 24 hours, medium was removed. Cells were treated with different concentrations of As₂O₃ or other drugs in a fresh medium. After 24, 48 or 72 hours, the medium in each well was removed and each well was washed with
PBS. Untreated control was prepared by treating cells in the medium only. Then, 30μl of MTT solution (5mg/ml) was added to each well and the plate was incubated at 37°C for 3 hours. After the incubation, the MTT was discarded and 100μl DMSO was added to dissolve the crystals in the cells and the culture was further incubated for 30 minutes at room temperature. DMSO (100μl) was used as blank. The absorbance at 540nm was measured using an ELISA plate reader (BIO-RAD).

Percentage survival of each sample was expressed using the following formula mean ± standard deviation (S.D.):

% Survival = 100% × (O.D. test sample/ O.D. control)

2.2.4 Detection of DNA Fragmentation

5×10^5 cells per well were seeded onto 60mm culture dish in 5ml RPMI 1640 medium and incubated at 37°C, 5% CO2. After the treatment, cells were lysed with 400 μl of DNA lysis buffer and vortexed until no cell debris was left. Twenty microliter of 10 mg/ml proteinase K was added and incubated at 37° for at least 2 hours. Then, 150μl of saturated NaCl was added, the sample was shake vigorously. The mixture was centrifuged at 6500×g for 15 minutes. The supernatant was collected, 1 ml of cold ethanol was added and centrifuge again at 15000×g for 20 minutes. After rinsed with cold 75% ethanol, the pellet was dried at room temperature. Finally, 20μl of 0.2mg/ml RNase A in Tris-EDTA buffer was added to each sample and further incubated at 37°C for 90 minutes. Equal amount of sample was electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining.
2.2.6 Flow Cytometry

2.2.5.1 Detection of Cell Cycle Pattern with PI

3×10^5 cells per well were seeded onto 6-well plate in 3ml RPMI 1640 medium and incubated at 37°C, 5% CO2. Twenty four hours later, medium was discarded and As2O3 in fresh medium was added. Then after 72 hours, cells were harvested and washed with PBS. Cells were then fixed with 1 ml of 70% ethanol at 4°C overnight. After fixation, the cells were centrifuged at 3000×g for 5 minutes to remove the ethanol. Cells were then resuspended in 0.46 ml freshly prepared propidium iodide solution containing propidium iodide (43 µg/ml) and RNase A (1mg/ml) and incubated in dark at 37°C for 30 minutes. After incubation, the cells were analyzed by FACSsort flow cytometer (Becton Dickinson).

2.2.5.2 Detection of Apoptosis with Annexin V-PI

The detection of apoptosis was performed by using BD Pharmingen™ Annexin V-FITC kit (Bio-Gene Technology Ltd.). 5×10^5 cells were seeded in a 60 mm culture dish. 24 hours later, medium was discarded and As2O3 was added with fresh medium. Then after 72 hours, cells were harvested and washed with PBS. 1×10^5 cells were used for the assay. 10µl of 10x binding buffer, 10µl of PI, 3µl of Annexin V-FITC conjugate and 77µl of dH2O were added to a sample and incubated in the dark at room temperature for 15 minutes. After incubation, 400µl of 1x binding buffer was added to the sample and the
sample was analyzed by FACSort flow cytometer (Becton Dickinson) as soon as possible. A computer program, WinMDI, was used for data analysis.

2.2.6 Western Blot Analysis

2.2.6.1 Protein Extraction

$1 \times 10^6$ cells were seeded on 100mm culture dish with 10ml RPMI 1640 medium. 24 hours later, As$_2$O$_3$ in the medium was added. Seventy two hours later, cells were collected and washed twice with PBS. 100μl of lysis buffer was added and allowed to stand on ice for at least 2 hours. Then, samples were boiled for 10 minutes and centrifuged at 13600×g for 10 minutes at 4°C. Finally, the supernatant was collected and stored at -20°C.

2.2.6.2 Protein Concentration Determination

The protein concentration was determined by bicinchoninic acid (BCA) protein assay. One microliter of each sample was mixed with 9μl of dH$_2$O in a 96-well plate. Bovine serum albumin (BSA) was used to draw the protein standard curve. Each sample and BSA standard was done in triplicate. BCA reaction mixture contained BCA solution and reagent B in the ratio of 50 : 1. 200μl of BCA mixture was added to each well and incubated at 37°C for 30 minutes. After incubation, the absorbance at 540 nm was measured by ELISA plate reader (Bio-Rad). Standard curve of protein amount was
plotted against the OD\textsubscript{540nm} (Fig. 2.1), and the amount of protein in the sample was calculated accordingly.

2.2.6.3 Western Blotting

**SDS-PAGE**

An apparatus of 3D vertical electrophoresis system (Bio-Rad) was assessed according to manufacturer's manual. Polyacrylamide separating gel (12.5%) was prepared with 1.0825 ml of dH\textsubscript{2}O, 1.6675 ml of 30% acrylamide, 1ml of 4× upper gel buffer, 4.65μl of TEMED and 20μl of 10% APS. 4.5% stacking gel was set with 1.2ml of dH\textsubscript{2}O, 0.3ml of 30% acrylamide, 0.5ml 4× lower gel buffer, 2.65μl of TEMED and 15μl of 10% APS.

According to the BSA standard curve, equal amount of protein samples (25-40μg) were used for SDS-PAGE. Protein samples were mixed with equal volume of 2× SDS loading dye and boiled for 10 minutes. The samples were loaded to the well of the polyacrylamide gel with rainbow coloured protein molecular marker (Amersham Bioscience Ltd.), and run at 120V for 90 minutes.

**Transfer of Protein to PVDF Membrane by Electroblotting**

Semi-Dry Electrophoretic Transfer Cell (semi-dry blotter, BioRad) was assessed according to the protocol as the manufacturer's manual. 0.45μm PVDF membrane (Millipore) was used for electroblotting. The dry PVDF membrane was soaked in 100% methanol for re-hydration. The membrane was then put in E-blot buffer for several
seconds. Three pieces of Whatman 3mm papers were soaked in E-Blot buffer and put onto the platinum anode. The membrane was put onto the Whatman 3mm papers and the gel was put onto the membrane. Another 3 pieces of E-blot buffer-soaked 3mm paper were then put onto the gel. The proteins were transferred at constant current at 0.15A for 2 gels for 60 minutes.

**Probing Proteins with Antibodies**

After transfer of protein to the PVDF membrane, the membrane was blocked with 10% non-fat milk (in TBS-T) at room temperature for 2 hours. Then, it was washed with TBS-T for 15 minutes for 3 times. Primary antibody, 0.6μl of mouse anti-human β-actin (as an example) in 3ml 10% non-fat milk was added for probing at room temperature for 2 hours. After probing, the membrane was washed with TBS-T for 10 minutes for 3 times and probed with secondary antibody (conjugated with horseradish peroxidase) at room temperature for 1 hour. The membrane was washed again with TBS-T for 10 minutes for 3 times and it was ready for ECL detection.

**Enhanced Chemiluminescence (ECL) Assay**

ECL assay was used for detection of probed proteins. ECL detection reagent 1 and reagent 2 (Amersham) were mixed in the ratio of 1:1 and the membrane was immersed in the mixture for 90 seconds. Then the membrane was wrapped and put into the Hypersensitive film cassette (Amersham) followed by a Fuji Medical x-ray film (Super
Rx, Fuji) in dark with various exposure time. The film was then developed by a film processor (M35 X-OMAT, Kodak).

2.2.7 In Vivo Study

2.2.7.1 Animal Model

Female nude mice as 4-6 week old were used for investigating the in vivo effect of As$_2$O$_3$ against breast cancer. The animals were housed under specific pathogen-free conditions in air-controlled rooms, which were specifically designed for maintenance of nude mice. Mice were fed with chow and sterile water ad lib in a 12-h light/dark cycle. To support the growth of the estrogen-dependent MCR-7 tumor, a 0.72-mg 17-β estradiol 60-day release pellet was implanted subcutaneously (s.c). Ten days before pellet inoculation, 1x10$^7$ cells were inoculated s.c. into the anterior part of the nude mice and allowed to grow for 5-10 days. The weights of the mice, size of tumor were measured before the treatment. Treatments were started when the tumor size reached about 50mm$^3$.

2.2.7.2 Treatment Schedule

The mice were divided into 3 groups, 7 mice per group, randomly. Groups included (1) PBS-control group, (2) 0.06mg/kg/day As$_2$O$_3$ group and (3) 0.12mg/kg/day As$_2$O$_3$ group. The dose of in vivo study was base on the results of in vitro study.

Nude mice were treated with As$_2$O$_3$ in PBS once every two days by intravenous injection. PBS-control group was injected with PBS only. The tumor size and body weight of the each mouse were measured before the treatment.
After a total 15 day treatment, the mice were sacrificed. The weight of the mice was recorded. All mice first anesthetized by intra-peritoneal (i.p.) injection of 0.1ml pentobarbital. Then, the mice were dissected and 0.5-0.8ml whole blood was withdrawn from the heart. The blood was transferred to a 1.5ml microfuge tube that contained 20μl of heparin and was centrifuged at 1000xg for 10 minutes at 4°C. After the centrifugation, the plasma was transferred to a new microfuge tube and stored at 4°C until enzyme assay was performed.

The volume of tumor was calculated as follows:

Volume of tumor (mm³) = length (mm) × width (mm) × height (mm)

Percentage of tumor growth

= volume of tumor (t) (mm³) / volume of tumor (f) (mm³) × 100%

where, volume of tumor (t) = the volume of tumor on day n after the treatment initiated

volume of tumor (f) = the volume of tumor on the day one

2.2.7.3 Toxicity of Arsenic Trioxide

To assess the toxicity of As₂O₃ after treatment, plasma enzymes activities were measured. After the plasma was collected, activities of four plasma enzymes, creatine kinase (CK), lactate dehydrogenase (LDH), alanine transaminase (ALT) and aspartate transaminase (AST), were measured by enzymatic diagnostic kits (Sigma). Increased activities of CK and LDH indicate heart tissue damage, while the increases of ALT and AST indicate liver tissue damage.

The procedures of enzymatic activities measurement were done according to the protocol of the enzymatic kits. Briefly, for measuring CK and LDH activities, 25μl of
plasma was mixed with 500 ml of CK or LDH reagent, respectively. The mixture was then incubated at 30°C for 3 minutes and 30 seconds for CK and LDH, respectively. The absorbance was measured at 340 nm by spectrophotometer (Beckman) for 2 minutes and 1 minute for CK and LDH, respectively at 30 seconds interval. For measuring AST and ALT, 100μl of plasma was mixed with 1 ml AST or ALT reagent and incubate at 37°C for 1 minute. Absorbance was measured at 340 nm at 30 seconds interval for 2 minutes.

The enzymatic activities were calculated as follows:

\[
\text{Enzymatic activity (U/L) = } \frac{\triangle A \text{ per min } \times TV \times 1000}{6.22 \times LP \times SV}
\]

where

- \(\triangle A \text{ per min}\) = change in absorbance per minute at 340 nm
- TV = total reaction volume (ml)
- LP = light path
- 6.22 = millimolar absorptivity of NADPH at 340 nm
- SV = sample volume
- 1000 = conversion of units per ml to per liter

thus,

\[
\text{CK (U/L) = } \triangle A \text{ per min } \times 8200
\]
\[
\text{LDH (U/L) = } \triangle A \text{ per min } \times 3376
\]
\[
\text{AST (U/L) = } \triangle A \text{ per min } \times 1768
\]
\[
\text{ALT (U/L) = } \triangle A \text{ per min } \times 1768
\]

The mean value of the plasma enzymatic activities of the same group was then plotted against the corresponding treatments.
Chapter 3
Anti-Proliferation effect of $\text{As}_2\text{O}_3$
on MDA-MB-231 cells
3.1 Study the Anti-proliferation Effect of As$_2$O$_3$ on MDA-MB-231 Cells by MTT Assay

Viable cells convert, MTT, from yellow colored salt to purple colored formazan, so the color changes indicate the percentage of cell survival after treatment.

In this part, we explore the potency of anti-proliferation effect of As$_2$O$_3$ on estrogen-independent cells line, MDA-MB-231 cells. MDA-MB-231 cells were cultured in complete RPMI 1640 medium as a monolayer. Then, the cells were treated with a range of concentrations of As$_2$O$_3$ prepared by serial dilute for 24, 48 and 72 hours. After the treatment, MTT assay was performed to assess the effect of cell survival of As$_2$O$_3$ on MDA-MB-231 cells. The results were shown in the Figure 3.1. The IC$_{50}$ of MDA-MB-231 cells treated with As$_2$O$_3$ were over 20µM, 18.4µM, 12.1µM for 24, 48, 72 hours respectively.
Fig 3.1 The anti-proliferate effect on MDA-MB-231 cells. MDA-MB-231 cells were treated with various concentrations of As$_2$O$_3$ for 24, 48 or 72 hours. Mean ± SD. n=4.
3.2 Comparison of Anti-proliferation Effect of As$_2$O$_3$ on MDA-MB-231 Cells to That of Tamoxifen

Tamoxifen, a non-steroidal antiestrogen, is the most commonly used therapeutic drug in the chemotherapy of breast cancer patients (Jaiyesimi et al., 1995). However, it has been reported that tamoxifen has little effect on treating estrogen receptor $\alpha$ negative breast cancers (Swain, 2001). The anti-proliferation effect of tamoxifen on MDA-MB-231 cells was also tested to compare with that effect of As$_2$O$_3$ by MTT assay.

MDA-MB-231 cells were incubated with a range of concentrations of tamoxifen or As$_2$O$_3$ for 48 and 72 hours. After treatment, the IC$_{50}$ of tamoxifen were 22.3$\mu$M, 18.3$\mu$M to MDA-MB-231 cells and IC$_{50}$ of As$_2$O$_3$ on MDA-MB-231 cells are 17.9$\mu$M, 12.4$\mu$M for 48 and 72 hours respectively (Fig 3.2).
Fig 3.2 Compare anti-proliferative effect of As$_2$O$_3$ and tamoxifen on MDA-MB-231 cells. MDA-MB-231 cells were treated with different concentrations of tamoxifen or As$_2$O$_3$ and (a) incubated for 48 hours. IC$_{50}$ of tamoxifen on MDA-MB-231 cells was 22.3µM, while the IC$_{50}$ of As$_2$O$_3$ was 17.9µM. (b) Incubated for 72 hours IC$_{50}$ of tamoxifen on MDA-MB-231 cells was 18.3µM, while IC$_{50}$ of As$_2$O$_3$ was 12.4µM. Mean ± SD. n=4.
3.3 Study Toxicity of \( \text{As}_2\text{O}_3 \) on Normal Breast Cells

Line, 184B5

Furthermore, 184B5 cells were used as a model to test the toxic effect of \( \text{As}_2\text{O}_3 \) on normal breast cells. 184B5 cells line was established from normal mammary tissue obtained from a normal reduction mammoplasty (Stampfer, 1989) and were cultured in complete MEGM medium (Lu et al., 2003).

As mentioned in section 1.5.1 \( \text{As}_2\text{O}_3 \) was considered as toxic agent for many centuries. The toxic effects caused by acute exposure to arsenic trioxide are caused by its ability to bind to cellular proteins containing sulfhydryl groups. Arsenic trioxide has direct toxic effects on endothelial cells, increasing the permeability of small blood vessels. A dose as little as 1 to 2.5 mg/kg of arsenic trioxide may cause people dead (Agency of Toxic Substances & Disease Registry). Although evidence from clinical trial indicates that \( \text{As}_2\text{O}_3 \) is harmless to patients with clinical dose and with careful monitor during treatment (Shen et al, 1997), it is necessary to investigate the toxicity of \( \text{As}_2\text{O}_3 \) on normal breast tissue.

The MTT assay was carried out to study the cytotoxicity effect of \( \text{As}_2\text{O}_3 \) on normal breast cancer cells, 184B5 (Fig 3.3).
Fig 3.3 Cytotoxicity effects of As₂O₃ on 184B5 and MDA-MB-231 cells. 184B5 and MDA-MB-231 cells were treated with different concentrations of As₂O₃ and (a) Incubated for 48 hours. IC₅₀ of As₂O₃ on 184B5 and MDA-MB-231 cells were 21.5μM and 17.9μM respectively. (b) Incubated for 72 hours IC₅₀ of As₂O₃ on 184B5 and MDA-MB-231 cells were 18.6μM and 12.4μM respectively. Mean ± SD. n=4
3.4 Summary

In this part, the anti-cancer effect of \( \text{As}_2\text{O}_3 \) on MDA-MB-231 cells was studied. According to the result of MTT assay, the \( \text{IC}_{50} \) of \( \text{As}_2\text{O}_3 \) treated MDA-MB-231 cells are large than 20\( \mu \text{M} \), 18.4\( \mu \text{M} \) and 12.1\( \mu \text{M} \) after incubated with drug for 24, 48 and 72 hours respectively which indicated that \( \text{As}_2\text{O}_3 \) can inhibit the proliferation of MDA-MB-231 cells in a dose and time dependent manner (Table 3.1).

\( \text{As}_2\text{O}_3 \) was more effective to the anti-tumor effect of tamoxifen on MDA-MB-231 cells, as the \( \text{IC}_{50} \) of \( \text{As}_2\text{O}_3 \) is lower than that of tamoxifen treated cells. (Table 3.2)

In addition, the cytotoxicity of \( \text{As}_2\text{O}_3 \) was investigated on human normal breast cells line, 184B5. The result demonstrated that MDA-MB-231 cells are more susceptible than the normal breast cells (Table 3.3).
Table 3.1 The IC$_{50}$ of As$_2$O$_3$ on MDA-MB-231 cells with 24, 48 and 72 hours treatment by MTT assay

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$ (μM)</td>
<td>&gt;20</td>
<td>18.3</td>
<td>12.1</td>
</tr>
</tbody>
</table>

Table 3.2 Compare the anti-proliferation effect of As$_2$O$_3$ and Tamoxifen on MDA-MB-231 cells for 48 and 72 hours treatment using IC$_{50}$.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>22.3</td>
</tr>
<tr>
<td>As$_2$O$_3$</td>
<td>17.9</td>
</tr>
</tbody>
</table>

Table 3.3 Compare the cytotoxicity of As$_2$O$_3$ on 184B5 and MDA-MB-231 cells for 48 and 72 hours treatment using IC$_{50}$.

<table>
<thead>
<tr>
<th>Cells Line</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>184B5</td>
<td>21.5</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>17.9</td>
</tr>
</tbody>
</table>
Chapter 4
Mechanism of Growth Inhibition
Effect of $\text{As}_2\text{O}_3$
on MDA-MB-231 cells
4.1 Cell Cycle Analysis of As$_2$O$_3$ Treated MDA-MB-231 Cells

PI is a red-fluorescent nuclear and chromosome counterstain that can be used to stain DNA. It is commonly used to analyse the apoptotic, necrotic and normal cells. When it is excited by 488nm laser beam, PI emits red light which will be detected by flow cytometry FL-2 channel. The intensity of fluorescence is proportional to the amount of PI bound on DNA. The amount of PI bound on DNA is related to the DNA contents of the cells and histogram can draw from the data by plotting the number of cells against the intensity of fluorescence. Then, we can estimate the percentage of cells in sub-$G_1$, $G_1$, $S$ and $G_2/M$ phase.

Analysis was performed for the MDA-MB-231 cells treated with different concentrations of As$_2$O$_3$ for 72 h. The result showed that there is an increase in sub-$G_1$ phase indicated that As$_2$O$_3$ may inhibit the growth of MDA-MB-231 cells by inducing apoptosis (Fig 4.1). An accumulation of cells in $S$ and $G_2/M$ phase observed from the results indicated that cell cycle arrest may also occur in the MDA-MB-231 cells (Fig 4.1 and Table 4.1).
Fig 4.1 Cell cycle of MDA-MB-231 cells treated with different concentration of As$_2$O$_3$ for 72 hours. $3\times 10^5$ cells were seeded in each well of 6-well plates. After treated with As$_2$O$_3$ for 72 h, the cells were stained with PI. The populations of cells in sub-G$_1$, G$_1$, S and G$_2$/M phase was determined by FACSort flow cytometer and analysed by WinMDI.
Table 4.1 The percentage of MDA-MB-231 cells distributions in sub-G1, G1, S and G2/M phase after treated with different concentrations of As2O3 for 72 hours

<table>
<thead>
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4.2 Detection of DNA Fragmentation

Apoptosis is known as programmed cells death. It can be characterized by changes in the plasma membrane, cell shrinkage, chromatin condensation and organized chromatin digestion to small fragments ("DNA ladders") (Bold et al., 1997). So the ability of As$_2$O$_3$ to induce apoptosis on MDA-MB-231 cells was examined by DNA fragmentation assay.

MDA-MB-231 cells were incubated with various concentrations of As$_2$O$_3$ for 72 hours. After treatment, the DNA contents were extracted and separated on 1.5% agarose gel. Ladder pattern was observed when the MDA-MB-231 cells were treated with 6.25μM, 12.5μM and 25μM As$_2$O$_3$ (Fig 4.2).
Fig 4.2 Result of DNA fragmentation assay of MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours. Lane M is the 100bp marker. Lane 1 is the control that cells incubated only with the medium. Lanes 2, 3, 4, 5 were cells treated with 3.125µM, 6.25µM 12.5µM and 25µM As$_2$O$_3$ respectively. A ladder pattern was observed under the concentration of 6.25µM, 12.5µM and 25µM.
4.3 Detection of Apoptosis Induced by As$_2$O$_3$ on MDA-MB-231 Cells by Flow Cytometry

Among the changes during the apoptosis, the changes in the plasma membrane are the earliest indicator. Annexin V is a Ca$^{2+}$-dependent phospholipids-binding protein. It binds to cells with exposed phospholipids phosphatidylserine (PS). In common, PS locates at inner leaflet of the plasma membrane. In the case of apoptosis, PS translocates to the outer leaflet of the plasma membrane and be bond by Annexin V. When Annexin V was conjugated to fluorochrome, fluorescein isothiocyanate (FITC) which emits green fluorescence, which identifies cells undergo apoptosis by flow cytometry FL-1 channel. However, we can not distinguish apoptotic cells from necrotic cells by using Annexin V alone, since the necrotic cells are also Annexin V positive. PI bind to DNA double helix. Early apoptotic cells have integrated membrane (PI negative) while late apoptotic and necrotic cells loss membrane integrity (PI positive). Therefore, PI was used to distinguish apoptotic cells from necrotic cells by flow cytometry FL-3 channel (Vermes et al., 1995).

After the MDA-MB-231 cells were treated, dot plots show an increase of percentage of apoptotic cells in a dose dependent manner (Fig 4.3).
Fig 4.3 The dot plots with the intensity of PI against Annexin V-FITC. 3×10^5 cells were seeded in each well of 6-well plates. After treated with As_2O_3 for 72 h, the cells were stained with PI and Annexin V-FITC. A shift of cell population to the right bottom quadrate was observed after As_2O_3 treatment.
4.4 Regulation of Apoptotic Related Protein by As$_2$O$_3$ on MDA-MB-231 Cells

Apoptosis is a process of deliberate life relinquishment by a cell in a multicellular organism. It is one of the main types of programmed cell death (PCD), and involves an orchestrated series of biochemical events leading to a characteristic cell morphology and death. The process of cell undergo apoptosis is controlled by many cell signals through both intrinsic (mitochondrial) and extrinsic (death receptor) pathways.

On one hand, apoptosis can be triggered by internal damage to the cell. For the intrinsic pathway, bcl-2 family proteins are involved. The proteins of the bcl-2 family can be divided into two parts: those of anti-apoptosis that are located on the surface of mitochondria and those of pro-apoptosis that are found in cytosol. They can react to the intercellular death signal and make holes on the mitochondrial membrane, causing cytochrome c to leak out (Reed et al., 1996; Amam and Cory, 2001). Cytochrome C will combine with other molecules to form aoptosomes to activate caspase-9, then other caspase which are known as cysteine proteases and one of the main executors of the apoptotic process (Hickman et al., 1996, Earnshaw et al., 1999).

On the other hand, apoptosis can also be triggered by external signals. The death receptors such as Fas ligand, TNF alpha (TNFα) play an important role in external pathway of apoptosis. These receptors are integral membrane proteins with their receptor domain exposed at the surface of the cell and can mediated apoptosis and active caspase cascade in a short time (Ashkenazi et al., 1999, Belka et al., 2000, Wajant, 2002).
In addition to the death receptor, bcl-2 family and caspase proteins, there are many other molecules related to apoptosis which are substrates of caspase: (1) The enzymes involved in DNA repair for example poly (ADP-ribose) polymerase (PARP). (2) The structural nuclear proteins, for example lamins which is degradation by caspase 6 during apoptosis (3) The enzymes caused DNA fragmentation such as caspase activated DNase (CAD)

In this part of the study, I use the Western blot analysis to examine the expression levels of apoptotic-related proteins to investigate apoptotic pathways involved in MDA-MB-231 cells induced by As$_2$O$_3$. 
Human bcl-2 protein is a 26 kDa, membrane-associate protein. It is one of the key regulators of apoptosis. It is an anti-apoptotic protein among bcl-2 family which can promote cell survival by interfering with the activation of mitochondrial-related apoptotic pathway and by interacting with other bcl-2 related family members (Reed, 1994).

Bax (B-9) is another member of Bcl-2 family. It is about 23kDa. Different from bcl-2, it is a pro-apoptotic protein. Overexpression of Bax will accelerates apoptotic death. It promotes apoptosis by competing with bcl-2 proper. Thus, the effects of bax and Bcl-2 suppress each other, so that high bcl-2/bax ratio suppresses apoptosis whereas low bcl-2/bax ratio triggers apoptosis (Adams and Suzanne, 1998).

The bcl-2 and bax protein expression levels were studied by western blotting. After 72 hours treatment, there is an obversely decrease in expression level of bcl-2 while there is no significant change on expression level of bax protein (Fig 4.4 and Fig 4.5).
Fig 4.4 Protein expression level of bcl-2 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-bcl-2 antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125μM, 6.25μM, 12.5μM and 25μM As$_2$O$_3$ respectively.
Fig 4.5 Protein expression level of bax in MDA-MB-231 cells treated with As₂O₃ for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-bax antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125µM, 6.25µM, 12.5µM and 25µM As₂O₃ respectively.
4.4.2 Expression Level of Cytochrome C

Cytochrome c or cyt c is a small heme protein found loosely associated with the inner membrane of the mitochondrion. The molecular weight of cytochrome C is about 11kDa. It is essential to energy conversion in all aerobic organisms. When it released from mitochondrion, it acts as a factor necessary for activation of apoptosis by forming caspase cascade (Liu et al., 1996). The release of cytochrome seems to be controlled by bcl-2 family members (Kluck et al., 1997).

The MDA-MB-231 cells were treated with various concentrations of As$_2$O$_3$ for 72 hours. Then, the western bolt analysis was performed. The result in the Fig 4.6 shows an increase of intensity in cytochrome c band with the increase of concentration of As$_2$O$_3$. 
Fig 4.6 Protein expression level of cytochrome c in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours. After 72 h treatment, cytosolic fraction proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-cytochrome c antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125μM, 6.25μM, 12.5μM and 25μM As$_2$O$_3$ respectively.
4.4.3 Expression Level of Caspase 9

Caspase is a family of cysteine-containing, aspartate-specific proteases. Caspase 9, also known as Apaf-3 is a 45 kDa protein exists as inactive proenzyme in cells. It is promoted by cytochrome c and catalyzed to the active form by forming a complex with Apaf-1 in the presence of dATP. Once caspase 9 is actived, it will promote the activation of other caspase such as the caspase 3, 6 and 7 (Earnshaw et al., 1999).

The expression level of caspase 9 was studied by western blot analysis. A mouse monoclonal anti-caspase-9 antibody was used to probe the sample. The pro-caspase 9 expression level was shown in the Fig 4.7. As expected, the protein level decreased in the drug treated MDA-MB-231 cells when compare to the control group.
Fig 4.7 Protein expression level of caspase 9 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-caspase-9 antibody (1:500). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125μM, 6.25μM, 12.5μM and 25μM As$_2$O$_3$ respectively.
4.4.4 Expression Level of FasL

Two important examples of the direct initiation of apoptotic mechanisms in mammals include the TNF (tumour necrosis factor) -induced model and the Fas-Fas ligand-mediated. Fas, which known as CD95, is a cell surface death receptor. Fas ligand (Fas L) is the ligand of Fas receptor with a molecular weight form 26 to 40 kDa. FasL binds with Fas receptor to form the death-inducing signaling complex, activates the caspase 8 and induce the apoptosis (Algeciras-Schimnich et al, 2002).

The expression level of Fas in MDA-MB-231 cells was studied by western blot analysis. The result was shown in the Fig 4.8. As expected, the protein level of FasL was upregulated in the drug treated MDA-MB-231 cells when compare to the cells treated only with medium.
Fig 4.8 Protein expression level of FasL in MDA-MB-231 cells treated with $\text{As}_2\text{O}_3$ for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-FasL antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125$\mu$M, 6.25$\mu$M, 12.5$\mu$M and 25$\mu$M $\text{As}_2\text{O}_3$ respectively.
4.4.5 Expression Level of Caspase 8

Human caspase 8 protein appears as a 50/55kDa doublet in cytosol. The capase 8 contains an aminoacid sequence enable it to interacte with Fas-associated death domain protein (FADD). FADD then acts as an adapter molecule and allow caspase 8 to become recruited to the cytoplasmic region of Fas following receptor activation. Caspase 8 is produced as a proenzyme which is activated by cleaving to smaller subunit (Cohen et al., 1997).

Studied by western blot analysis, the antibody recognizes the proform (50/55kDa doublet) of human caspase-8. (Fig 4.9) And the expression level of pro-caspase 8 decreased significantly in the MDA-MB-231 cells treated with As$_2$O$_3$ when compare with the untreated cells.
Fig 4.9 Protein expression level of caspase 8 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-caspase-8 antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125μM, 6.25μM, 12.5μM and 25μM As$_2$O$_3$ respectively.
4.4.6 Expression Level of Caspase 3

Caspase 3, also known as CPP32, apopain and Yama, is one of the key mediator molecules of apoptosis. After the inactive 32kDa pro-caspase 3 is cleaved to the 17 or 11 kDa active form by other family members of caspase, for example caspase8, it will in turn be responsible for the cleavage of other key cellular proteins such as Poly (ADP-ribose) polymerase (PARP) and leads to apoptosis. Besides its role in activation of cellular proteins, it can also activate other caspase: caspase 6, 7 and 9 (Cohen et al., 1997).

The expression level of caspase 3 protein was studied by western blotting. After 72 hours treatment, there is an obversely decrease in expression level of pro-caspase-3 in the As$_2$O$_3$ treated MDA-MB-231 cells to the cells treated only with RPMI medium. The result of the expression level of caspase 3 by western blotting was shown in Fig 4.10.
Fig 4.10 Protein expression level of caspase 3 in MDA-MB-231 cells treated with \( \text{As}_2\text{O}_3 \) for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-caspase-3 antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125\( \mu \)M, 6.25\( \mu \)M, 12.5\( \mu \)M and 25\( \mu \)M \( \text{As}_2\text{O}_3 \) respectively.
4.4.7 Expression Level of Poly (ADP-ribose) Polymerase (PARP)

PARP involves DNA repair and programmed cell death. It is the first identified caspase substrate protein. During the apoptosis, caspase 3 cleaves the 116 kDa into 85 kDa active form (Tewari et al., 1995).

The expression level of PARP in MDA-MB-231 cells was studied by western blot analysis. PARP (H-250), a rabbit polyclonal anti-PARP antibody was used to probe the sample. The proform PARP protein expression level was shown in the Fig 4.11. As expected, the protein level decreased in the drug treated MDA-MB-231 cells when compare to the control group.
Fig. 4.11 Protein expression level of PARP in MDA-MB-231 cells treated with \(\text{As}_2\text{O}_3\) for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-PARP antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125\(\mu\text{M}\), 6.25\(\mu\text{M}\), 12.5\(\mu\text{M}\) and 25\(\mu\text{M}\) \(\text{As}_2\text{O}_3\) respectively.
The tumor suppressor gene p53 plays an essential role in surveillance of DNA damage, and in regulation of the cell cycle and apoptosis. The wild-type p53 gene is essential for regulation of cell growth and loss of p53 function may be involved in the early steps of tumor formation through the survival of cells with genetic mutations. Oren et al. reported in 1994 that p53 can arrest cells in the G1 phase of cell cycle to allow the DNA damage to repair, however, if the damage too serious to be repaired, it will induce apoptosis of the cells (Oren, 1994). A number of human cancers, including lung, colon have been shown to mutate in the p53 gene. Tumors that possess either mutation or deletion of the p53 gene are still capable of undergoing apoptosis, such as in breast cancer upon exposure to Taxol (Strobe1 et al., 1996). It also has been demonstrated that p53 is capable of stimulating bax expression (Miyashita and Reed, 1995).

Investigated by western blot analysis, the antibody recognizes 53 kDa human p53. (Shown in Fig 4.12) And the expression level of p53 is downregulated in the MDA-MB-231 cells treated with As$_2$O$_3$ when compare with the untreated cells.
Fig. 4.12 Protein expression level of p53 in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-p53 antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125µM, 6.25µM, 12.5µM and 25µM As$_2$O$_3$ respectively.
4.5 Regulation of Cell Cycle Related Protein by As$_2$O$_3$ on MDA-MB-231 Cells

The cell cycle is the series of events that take place in a cell between its formation and its replication. Most cells have a typical four phase cell cycle: G$_1$, S, G$_2$ and M phase. Some cells may temporarily or reversibly stop division and enter a state of quiescence called G$_0$ phase.

Cell cycle is a complicated process and it is controlled by many regulators. Regulators work on the checkpoint of cell cycle process. At the end of the G1 and G2 phase, and after DNA replication in the S phase, it is checked for damages. At the end of the M phase a checkpoint stop cytokinesis, if the chromosomes are not properly aligned on the mitotic spindle. There are two main kinds of regulators involving in the cell cycle: cyclins and cyclin-dependent kinases (cdks). Cyclins bind to cdks to form cyclin-cdk complexes that regulates the cell cycle. The main types of cyclin-cdk complexes include cyclin D-cdk4/6 for G$_1$ phase, cyclin E-cdk2 for G$_1$ to S phase transition, cyclin A-cdk2 for S phase, cyclin A/B-cdk1 for G$_2$ and M phases (Murray and Kirschner, 1991). As mentioned in the section 4.4, p53 block the cell cycle at the checkpoint of G$_1$ phase to allow the cell to repair DNA (Lee and Bernstein A, 1995).
4.5.1 Expression Level of Cyclin B

Cyclin B is a mitotic cyclin with a molecular weigh of 58 kDa. In mammals cyclin B is involved in G2/M phase transitions of the cell cycle associate with cdk1. The complex remains inactive until the completion of DNA synthesis and then activated by a specific protein phosphatase, Cdc 25. The amount of cyclin B (which binds to Cdk1) and the activity of the cyclin B-Cdk complex rise through the cell cycle until mitosis, when they fall abruptly due to degradation (Dunphy, 1994).

As shown by the cell cycle analysis by PI staining (Table 4.1), there was an increase in percentage of cells in G2/M phase after MDA-MB-231 cells are treated with different concentration of As2O3, indicating that As2O3 may induce a cell cycle arrest at G2/M phase. The expression level of cyclin B was explored by western blotting. The result was shown in Fig 4.13. There is a marked increase in expression level of Cyclin B with the increasing concentration of As2O3.
Fig. 4.13 Protein expression level of cyclin B1 in MDA-MB-231 cells treated with 
As$_2$O$_3$ for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal 
amount of each sample were resolved by SDS-PAGE and probed with anti-cyclin B1 
antibody (1:500). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125μM, 6.25μM, 12.5μM and 25μM As$_2$O$_3$ respectively.
4.5.2 Expression Level of Cyclin E

Cyclin E is expressed during G1 phase with a reduced molecular weight of about 50 kDa. Cyclin E associated with cdk2 specifically regulates the transition from the G1 phase to the S phase of the cell cycle. A high level of cyclin E accelerates the transition of the cell through the G1 phase; however, p53 tumor suppressor gene inhibits the cyclin E-cdk2 activity after DNA damage (Di Leonardo et al., 1994).

Lindahl et al reported recently that cyclin E is of prognostic value in breast cancer. High levels of cyclin E in the tumor correlate with an aggressive tumor type and poor survival of the patients (Lindahl et al, 2007).

The cyclin E expression level was studied by western blotting. After 72 hours treatment, there is no significant change on expression level of cyclin E protein (Fig 4.14).
Fig. 4.14 Protein expression level of cyclin E in MDA-MB-231 cells treated with As$_2$O$_3$ for 72 hours. After 72 h treatment, total cellular proteins were extracted. Equal amount of each sample were resolved by SDS-PAGE and probed with anti-cyclin E antibody (1:100). Lane 1 is the control that cells incubated only with medium. Lanes 2, 3, 4, 5 were cells treated with 3.125µM, 6.25µM, 12.5µM and 25µM As$_2$O$_3$ respectively.
4.6 Summary

The results of the mechanism study indicated that As$_2$O$_3$ inhibit the proliferation of MDA-MB-231 cells via induction of both cycle arrest and apoptosis which were shown in the results of cell cycle analysis (Fig 4.1, Table 4.1, Fig 4.2 and Fig 4.3).

The results of western bolt analysis further confirmed the former results that:

(1) As$_2$O$_3$-induced apoptosis was dependent on the extrinsic pathway, as shown by increase of FasL expression level (Fig 4.8), caspase 8 (Fig 4.9), caspase 3 (Fig 4.10) and PARP (Fig 4.11) activation.

(2) As$_2$O$_3$-induced apoptosis was dependent on the mitochondria intrinsic pathway, as shown by activation of caspase 9 (Fig 4.7), what’s more, the increase of cytochrome c expression (Fig 4.6) and the decrease of bcl-2 expression, thus the increase ratio of Bax/bcl-2 (Fig 4.4 and Fig 4.5).

(3) As$_2$O$_3$ induced cell cycle arrest at G$_2$/M phase, as shown by increase of cyclin B expression (Fig 4.13), whereas it did not induced G$_1$ arrest for there is no change on cyclin E expression (Fig 4.14).

(4) The tumor suppression gene p53 is not involved in the anti-proliferation effect of As$_2$O$_3$ as shown in the Fig 4.12 that the expression level of p53 is downregulated in the As$_2$O$_3$ treated MDA-MB-231 cells.
Chapter 5

*In Vivo* Study of Anti-tumor Effect of $\text{As}_2\text{O}_3$
5.1 Anti-tumor Effect of As$_2$O$_3$ on Tumor Bearing Nude Mice

In the previous study, As$_2$O$_3$ has found to inhibit the growth of breast cancer cells *in vitro* by inducing apoptosis and cell cycle arrest. The anti-proliferation effects of As$_2$O$_3$ can be observed both on ER$\alpha$ positive and ER$\alpha$ negative breast cancer cells lines. I next investigate the anti-tumor and possible toxic effects of As$_2$O$_3$ on nude mice.

Four to six week female nude mice bearing tumor were used as the animal model. Each mouse was inoculated $1 \times 10^7$ MCF-7 cells in PBS. A 0.72mg, 60-day releasing 17$\beta$-estradiol pellet (Innovative Research of America) was inoculated s.c. into the anterior part before inoculation of estrogen dependent MCF-7 cells. After the mice developed tumor, the mice were divided into 3 groups: PBS-control group, 0.06mg/kg/day As$_2$O$_3$ group and 0.12mg/kg/day As$_2$O$_3$ group. Nude mice were treated with As$_2$O$_3$ in PBS every two days and the body weight and tumor size were measured during the treatment and calculated after treatment.

The results show a decrease of tumor growth in nude mice of As$_2$O$_3$ treatment group (Fig 5.1) and during the treatment the body weight of the mice remains constantly (Fig 5.2). The t-Test was carried out by the Microsoft Office Excel software to compare the significance between the results of each two treatment groups.
Fig 5.1 The effect $\text{As}_2\text{O}_3$ on the tumor growth in tumor bearing nude mice. The tumor size of the nude mice are measured by using electronic caliper and calculated with a formula: tumor size$(\text{mm}^3) = \text{length (mm)} \times \text{width (mm)} \times \text{height (mm)}$ every two days during treatment. The percentage of tumor growth was shown by compare the tumor size of the later days with the tumor size of the first day treatment. The first day treatment is noted as day 1. Data were mean± S.D., n=7. t-Test was performed: $p<0.05$ between control group and 0.06mg/kg/day $\text{As}_2\text{O}_3$ group; $p<0.02$ between control group and 0.12mg/kg/day $\text{As}_2\text{O}_3$ group.
Fig 5.2 The changes of body weights of tumor bearing nude mice during treatment. The body weigh of nude mice were measured every two days during treatment with different concentration of As$_2$O$_3$. The first day treatment is noted as day 1. Data were mean± S.D., n=7.
5.2 Toxic Effect of As$_2$O$_3$ on Normal Tissues

Among the large amount of enzymes in the body, some of them are tissue specific. In normal situations, the level of these tissue specific enzymes is low in the plasma. When there is damage to the tissue which causes the tissue cell death, the enzymes will be released into the plasma. So measuring the activity of these tissue specific enzymes in the plasma enables us to detect whether there is damage to the related tissue.

The side effects of As$_2$O$_3$ are mainly report on the cardiac toxicity. The heart and liver are the two main tissue of the body. Activity of four enzymes in plasma was measured to study toxicity of As$_2$O$_3$ on normal cells: Aspartate dehydrogenase (AST) which located mainly in liver, heart and skeletal; Alanine dehydrogenase (ALT) which located mainly in liver, kidney and heart; Creatine kinase (CK) which located mainly in heart and Lactate dehydrogenase (LDH) which is located mainly in heart. So the cardiac toxicity of As$_2$O$_3$ can be assessed by measurement of plasma level of CK and LDH, while the hepatic toxicity by ALT and AST (Lo et al., 2002).

After 15 days treatment the nude mice were sacrificed and the blood was collected from the heart. The plasma was separated for enzyme activity analysis following the protocols of the enzymatic kits. There was no significant change of AST and ALT activities in the plasma (Fig 5.3 and Fig 5.4) when compared with that of the PBS control after As$_2$O$_3$ treatment for 15 days.

For the cardiac toxicity of As$_2$O$_3$, there is a slight increase of CK and LDH activities in the plasma (Fig 5.5 and Fig 5.6), when compared with that of the PBS control. During 15 day treatment, no death and hemolysis occurred in all groups.
Fig 5.3 Effect of $\text{As}_2\text{O}_3$ on Aspartate dehydrogenase (AST) activity of nude mice. PBS, 0.06mg/kg/day and 0.12mg/kg/day $\text{As}_2\text{O}_3$ was injected intravenously into nude mice every two days for 15 days. After treatment, the mice were sacrificed and the plasma was collected to perform enzyme assays. Normal mice injected with PBS were used as control for comparison. Data were mean± S.D., n=7.
Fig 5.4 Effect of As$_2$O$_3$ on Alanine dehydrogenase (ALT) activity of nude mice. PBS, 0.06mg/kg/day and 0.12mg/kg/day As$_2$O$_3$ was injected intravenously into nude mice every two days for 15 days. After treatment, the mice were sacrificed and the plasma was collected to perform enzyme assays. Normal mice injected with PBS were used as control for comparison. Data were mean±S.D., n=7.
Fig 5.5 Effect of As$_2$O$_3$ on Creatin Kinase (CK) activity of nude mice. PBS, 0.06mg/kg/day and 0.12mg/kg/day As$_2$O$_3$ was injected intravenously into nude mice every two days for 15 days. After treatment, the mice were sacrificed and the plasma was collected to perform enzyme assays. Normal mice injected with PBS were used as control for comparison. Data were mean± S.D., n=7.
Fig 5.6 Effect of As$_2$O$_3$ on Lactate dehydrogenase (LDH) activity of nude mice. PBS, 0.06mg/kg/day and 0.12mg/kg/day As$_2$O$_3$ was injected intravenously into nude mice every two days for 15 days. After treatment, the mice were sacrificed and the plasma was collected to perform enzyme assays. Normal mice injected with PBS were used as control for comparison. Data were mean± S.D., n=7
5.3 Summary

In vivo study showed As$_2$O$_3$ was able to inhibit the breast tumor growth in tumor bearing nude mice in a dose dependent manner (Fig 5.2).

The enzyme activity analysis showed no significant increase of plasma level of specific tissue enzymes, AST, ALT, CK and LDH after 15 days treatment (Fig 5.3, 5.4, 5.5 and 5.6). These results, together with the constant body weight of the nude mice (Fig 5.1) indicated As$_2$O$_3$ dose not cause serious damage to then animal at the dose of 0.12mg/kg/day.
Chapter 6
Discussion
6.1 Anti-tumor Effect of As$_2$O$_3$ on Breast Cancer

In addition to the significant anti-cancer effect on APL, As$_2$O$_3$ has been studied on its potential use in other types of Leukemia as well as solid tumor (Konig et al., 1997, Ling et al., 2002, Li et al., 2004). In the previous study of our group, As$_2$O$_3$ was found to be able to inhibit the proliferation of ER$\alpha$ positive MCF-7 cells in vitro. Further mechanism study indicated As$_2$O$_3$ induced apoptosis and cell cycle arrest on MCF-7 cells. And these effects were related to the ability of As$_2$O$_3$ in regulating the ER$\alpha$ signaling pathway (Chow et al., 2004).

Women patients of breast cancer at late stage always have their tissue contain ER$\alpha$ negative cells. To study the anti-tumor effect of As$_2$O$_3$ on late stage breast cancer, MDA-MB-231 cells line, an ER$\alpha$ negative breast cells line, was used to mimic the late stage of breast cancer in this study. The result indicated that the As$_2$O$_3$ can inhibit the MDA-MB-231 cells growth in a dose and time dependent manner (Fig 3.1 and Table 3.1) and inhibited the MDA-MB-231 cells growth at a much lower concentration than the tamoxifen did (Fig 3.2 and Table 3.2).

Pharmacokinetics study performed by the Shen et al. during the treatment of APL suggested that it is safe to inject As$_2$O$_3$ with a dose of 10mg/day for the plasma arsenic was rapidly eliminated and continuous administration of As$_2$O$_3$ did not result in the accumulation of arsenic in plasma (Shen et al., 1997). Our data on nude mice showed that As$_2$O$_3$ at concentration of 0.06mg/kg/day to 0.12mg/kg/day has no appeared toxicity on the animal.
6.2 Induction of Apoptosis and Cell Cycle arrest by AS$_2$O$_3$

Induction of apoptosis and cycle arrest are the most popular mechanisms studied on the chemotherapy drug these years. AS$_2$O$_3$ was reported to induce apoptosis in a wide range of cancer cell lines (Shao et al., 1998, Zhu et al., 1999, Kinjo et al, 2000, Li et al., 2004., Chan et al., 2006). Apart from apoptosis, regulation of cell cycle is the other important mechanism involve in the anti-cancer effect AS$_2$O$_3$ (Lin et al., 2000, Park et al., 2003, Wu et al., 2004, Shao et al., 2005). In our study, AS$_2$O$_3$ at concentration 3.125µM over induced apoptosis in MDA-MB-231 cells. This is confirmed by a typical DNA ladder pattern appeared in MDA-MB-231 cells appeared during DNA fragmentation assay (Fig 4.2). To further confirm this result, we tested the externalization of PS by Annexin-V and PI staining. An increase in apoptotic cell was observed after the AS$_2$O$_3$ treatment (Fig 4.3). Together with the detection of sub-$G_1$ phase increase in cell cycle analysis (Fig 4.1 and Table 4.1), it was suggested that AS$_2$O$_3$ induced apoptosis in MDA-MB-231 cells. Meanwhile, the increase of MDA-MB-231 cell population in G$_2$/M phase indicated the AS$_2$O$_3$ induced G$_2$/M cell cycle arrest in MDA-MB-231 cells (Fig4.1 and Table 4.1).

In previous study, AS$_2$O$_3$ has been found to induce apoptosis in different human carcinoma cell lines by regulating the expression level of apoptotic related protein, however mechanisms under the AS$_2$O$_3$ induced apoptosis is quite different. Chow et al. reported AS$_2$O$_3$ induced apoptosis in ER$\alpha$ positive breast cancer cell line MCF-7 was due to the collapse of mitochondrial membrane potential, up-regulation of tumor suppressor
gene p53 and regulation of ERα signaling pathway (Chow et al., 2004). As the MDA-MB-231 cells are known to be ERα negative, the effect of As2O3 on it does not involve ERα. And the features of MCF-7 cells and MDA-MB-231 cells are quite different from each other. For example, caspase 3 is a key factor for initiation of DNA fragmentation. DNA fragmentation was not detected during apoptosis in MCF-7 cells for that MCF-7 cells do not express caspase 3 gene (Kurokawa et al., 1999), while MDA-MB-231 cells express caspase 3 (Yang et al., 2003) with DNA fragmentation occurring during apoptosis. In our study, the involvement of extrinsic apoptotic pathway in the apoptosis in MDA-MB-231 cells induced by As2O3 was confirmed as the expression level of Fas receptor Ligand (FasL) increased (Fig 4.8) and the activation of caspase 8 (Fig 4.9), while an increase ratio of bax/bcl-2 (Fig 4.4 and Fig4.5), followed by increasing level of cytochrome c (Fig 4.6) and activation of caspase 9 (Fig 4.7) suggested the involvement of intrinsic apoptotic pathway. p53 is involved in both cell cycle and apoptosis. It was found to be upregulated and induce apoptosis through regulating bax (Miyashita and Reed, 1995, Zhao et al., 2000). From our study, the expression level of p53 is decreased (Fig 4.12) which indicated the p53 did not directly trigger the apoptosis in MDA-MB-231 cells. Since the function of p53 is let the cell repair from DNA damage, lacking of p53 may cause the prolong DNA damage in the MDA-MB-231 cells. And then the DNA damage may activate the intrinsic apoptotic pathway by down-regulating the expression level of bcl-2 (Fig 4.4).

The induction of cell cycle arrest on various cancer cell lines by As2O3 is mostly related to the arrest in G1 and G2/M phase (Lin et al., 2000, Park et al., 2003, Wu et al., 2004, Chow et al., 2004, Shao et al., 2005). Cyclin B1 associate with cdk1 and remains
inactive in cytosol until it is activated by phosphorylation (McFowan and Russell, 1995) and cyclinB-cdk1 induced G2/M phase arrest by prevention of the dephosphorylation of cyclinB-cdk1 complex (Halloran and Fenton, 1998, Yin et al., 2001). In my study, the changes in expression levels of cyclin-B1 and p53 indicate that the As2O3 inducing G2/M phase cell cycle arrest via prevention of the dephosphorylation of cyclinB-cdk1 complex for the increase of cyclin-B1 (Fig 4.13) and decrease of p53 (Fig 4.12) expression. Cyclin E-cdk2 regulates the transition from G1 to S phase and p53 tumor suppressor gene inhibits the cyclin E-cdk2 activity after DNA damage (Lang et al., 2003, Di Leonardo et al., 1994). In my study, there is an significant decrease in percentage of MDA-MB-231 cells in G1 phase (Table 4.1), together with the decrease of p53 expression (Fig 4.12) and no change of cyclin E expression in As2O3 treated MDA-MB-231 cells in comparison to the control group, indicate that the p53 may not involve in the As2O3 induction of cell cycle arrest for that p53 in the MDA-MB-231 cells may be non-functional or mutated (Hui et al., 2006).

6.3 Side Effect of As2O3 on Breast Cancer Treatment

For thousands of year, As2O3 was considered as a toxic agent in both Chinese and western society. Recent clinical use of As2O3 in treating APL reported the possible adverse effect during treatment. The side effect of As2O3 mainly detected in the heart disorders, for instance, the QT prolongation, torsade de pointes tachycardias (TdP), sudden death from heart attack (Zhang et al., 2001, Zhou et al., 2003, Ficker et al., 2004). The growth inhibition of As2O3 on 184B5 cells was studied to investigate the toxic effect of As2O3 on normal breast tissue. When the As2O3 inhibited 50% MDA-MB-231 cells,
the percentage of survival of 184B5 cells was over 70% for 48 and 72 hours As₂O₃ treatment. And in a lower concentration of As₂O₃ less than 12.5μM, MDA-MB-231 cells were more susceptible to the As₂O₃ than the normal breast cells. In addition, the toxic effect of liver and heart tissues were studied in vivo on two main tissues in the body: heart and liver. The dosage of As₂O₃ used is equivalent to that in in vitro study of MCF-7 cells (data not shown) and is less than the clinical application of the drug that the results suggested it was safe to be a medicine. In the study, we tested four tissue specific enzymes; two are located mainly in liver and two are located mainly in heart. There are no significant changes in plasma level of four enzymes (Fig 5.3, 5.4, 5.5 and 5.6), showing there is no toxic effect of As₂O₃ on both tissues with the dosage of 0.06mg/kg/day and 0.12mg/kg/day As₂O₃ treatment.

In conclusion, As₂O₃ was effective in treating breast cancers both early stage and late stage. The present study also provided promising data on the possible application of As₂O₃ on breast cancer cells which are ERα negative.
Chapter 7
Future Perspectives
7.1 Future Prospectives

Although we found that the As$_2$O$_3$ inhibits the tumor growth through induction of apoptosis and cell cycle arrest, the detailed mechanisms were not fully investigated in this study. Apoptosis is a very complicated process. Apart from the intrinsic pathway and extrinsic pathway of apoptosis, other molecules and pathways may also involve in it, for example the other members of bcl-2 family and caspase family. More cyclin and cdk should be studied as well as other cell cycle related proteins. There have been similarities between progression through cell cycle and induction of apoptosis. Many regulators have effect on both progresses. For example, the ErbB2 antisense in breast cancer, which is particularly over-expressed in breast carcinoma, have effect both on cell-cycle activation and on apoptosis in the breast cancer (Bold et al., 1997, Makin and Dive 2001). As the p53 found to have no direct effect on apoptosis and cell cycle arrest in MDA-MB-231, the mechanism cause the ineffective of p53 should also be studied. More experiments should be performed to get a full understand on mechanisms of As$_2$O$_3$ induced apoptosis and cell cycle arrest.

It has been reported that the anti-tumor effect As$_2$O$_3$ on ER$\alpha$ positive breast cancer cell line MCF-7 included the ability of As$_2$O$_3$ to regulate the ER$\alpha$ signaling pathway (Chow et al, 2004). Although the MDA-MB-231 cells are ER$\alpha$ negative, it expresses ER$\beta$ on its surface and is defined as ER$\beta$ positive. The ER$\alpha$ and ER$\beta$ are the two forms of estrogen receptor and have the similar structure with each other. It is reasonable to investigate whether As$_2$O$_3$ have effect on the ER$\beta$ signaling.
As the breast cancers are solid tumor, As$_2$O$_3$ can be directly injected s.c. at the site adjacent to the tumor. Without diluted by blood, the concentration of drug will be high for a period of time at tumor side after s.c. injection. In Comparison to i.v. injection, the amount of drug enters into the blood is lower than injected s.c. This will reduce the side effect on other tissues caused. Finally, the As$_2$O$_3$ have anti-proliferation effect on both MCF-7 and MDA-MB-231 cells in vitro, the mechanisms of the As$_2$O$_3$ effect are not the same for the two cell lines. In vivo anti-tumor effect of As$_2$O$_3$ on tumor bearing nude mice with MDA-MB-231 cells should be investigated to confirm the in vivo anti-tumor effect of As$_2$O$_3$ on advanced stage breast cancer.
Reference


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