Novel mechanism of 2DG mediated cancer treatment /Zhang Shiqing.

Shiqing Zhang

Follow this and additional works at: https://repository.hkbu.edu.hk/etd_oa

Recommended Citation
https://repository.hkbu.edu.hk/etd_oa/353

This Thesis is brought to you for free and open access by the Electronic Theses and Dissertations at HKBU Institutional Repository. It has been accepted for inclusion in Open Access Theses and Dissertations by an authorized administrator of HKBU Institutional Repository. For more information, please contact repository@hkbu.edu.hk.
DATE: August 29, 2016

STUDENT'S NAME: ZHANG Shiqing

THESIS TITLE: Novel Mechanism of 2DG Mediated Cancer Treatment

This is to certify that the above student's thesis has been examined by the following panel members and has received full approval for acceptance in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Chairman: Prof Yang Sze-Ming Mildred
Professor, Division of Science and Technology, UIC
(Designated by Dean of Division of Science and Technology, UIC)

Internal Members: Dr Wu Mei Yi Daphne
Assistant Professor and Acting Programme Director, Food Science and Technology, UIC

Dr Lei Bo
Associate Professor, Food Science and Technology, UIC

External Members: Prof Jin Dong-Yan
Professor
School of Biomedical Sciences
The University of Hong Kong

Prof Cho Chi Hin
Professor
School of Biomedical Sciences
The Chinese University of Hong Kong

In-attendance: Prof Chung Sum Man
Dean & Professor, Division of Science and Technology, UIC

Issued by Graduate School, HKBU
Novel Mechanism of 2DG Mediated Cancer Treatment

ZHANG Shiqing

A thesis submitted in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

Principle Supervisor: Prof. Stephen Chung
Hong Kong Baptist University

August, 2016
DECLARATION

I hereby declare that this prospectus represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis, dissertation submitted to this or other institution for a degree, diploma or other qualification.

Signature: 张世卿

Date: August 2016
Abstract

2-deoxy-D-glucose (2DG), a non-metabolizable glucose analog, is currently being used in clinical trials to determine its efficacy in augmenting radiotherapy and chemotherapy of various cancers. It is thought to kill cancer cells by inducing glucose deprivation state. However, 2DG only inhibits glycolysis by 15-40%, not sufficient to simulate glucose deprivation. Further, 2-flour-deoxy-D-glucose (2FDG), which is a more potent inhibitor of glycolysis than 2DG, is less effective than 2DG in killing cancer cells. These observations suggest that glucose deprivation is not the mechanism by which 2DG kills cancer cells.

On the other hand, it has been shown that treatment of cancer cells with 2DG leads to increased reactive oxygen species (ROS) production, indicating that cytotoxic effect of 2DG is due to increase ROS. Our lab previously found that inhibition of aldose reductase (AR) activity attenuated 2DG-induced ROS in cardiomyocytes (Tang, et al., 2010). We therefore propose that 2DG-induced ROS increase in cancer cells is a consequence of the depletion of GSH in the process of reduction of 2DG by AR and/or by a related aldose reductase-like enzyme (ARL). These two enzymes are often overexpressed in cancer cells. This proposed project is to test our hypothesis that the cytotoxicity of 2DG is due to the depletion of GSH as a consequence of the reduction of 2DG by AR or ARL.

We found that HepG2, SKOV3, HCT116 and CaCo2 cells were sensitive to 2DG, and these cells over-express AR and/or ARL proteins. However, HT29 cells and SW480 cells, which were not sensitive to 2DG, had low level of AR and ARL proteins,
indicating that there is a close relationship between sensitivity to 2DG toxicity and the level of AR/ARL in these cells. Further, when AR/ARL activity were inhibited in HepG2, SKOV3, HCT116 and CaCo2 cells by AR/ARL inhibitors fidarestat or tolrestat, the cells were protected against 2DG cytotoxicity. Tolrestat or fidarestat significantly restored the drop of GSH levels in 2DG sensitive cancer cells induced by 2DG. On the other hand, MG-132 and bortezomib, which increased the expression of AR/ARL in HT29 and SW480 cells, made HT29 and SW480 cells more sensitive to 2DG. These experiments confirmed our hypothesis that 2DG toxicity in cancer cells was due to oxidative stress induced by AR/ARL.

2DG is not an efficient substrate for AR/ARL enzymes and it is not very efficient in killing cancer cells. Based on our hypothesis, better AR/ARL substrates should be more toxic to cancer cells that overexpress AR/ARL than 2DG. The cytotoxic effects of glyceraldehyde and diacetyl, which were better substrates for AR/ARL than 2DG, were tested. Both glyceraldehyde and diacetyl were more efficient in killing cancer cells that over-express AR and/or ARL (HepG2, SKOV3, HCT116 and CaCo2) than cancer cells with low levels of AR and ARL proteins (HT29 and SW480). Glyceraldehyde and diacetyl were more efficient in lowering the GSH level in cancer cells that over-express AR and/or ARL. In order to further develop glyceraldehyde and diacetyl as anti-cancer drugs, animal studies were carried out to determine their anti-cancer effects. Both glyceraldehyde and diacetyl significantly inhibited the tumor growth in nude mice tumor xenograft model.

In conclusion, this thesis proposed and proved that 2DG kills cancer cells by
lowering intracellular GSH levels as a consequence of its reduction by AR/ARL activities, rather than by inhibition of glycolysis. This novel mechanism predicts that better substrates for AR/ARL than 2DG would be more effective in killing cancer cells than 2DG. This was confirmed by using glyceraldehyde and diacetyl. We believe that this would lead to the development of more efficient anti-cancer drugs.

**Key words:** 2DG, Polyol pathway, AR, ARL, Oxidative stress, Glyceraldehyde, Diacetyl, Cancer-specific cytotoxicity.
ACKNOWLEDGMENTS

I would like to express my sincere appreciation to various people who have helped and encouraged me during my PhD study in UIC and Hong Kong Baptist University.

First and foremost, I would like to express my sincere appreciation to my supervisor Prof. Stephen Chung for providing me with valuable guidance and excellent advice, and for his continuous encouragement and great help both in my study and in my career development. In addition, I am also indebted to my co-supervisor Prof. Ken Yung for his patient guidance and generous supports during my study. It is a great honor for me to be their student.

Furthermore, I would like to express my heartfelt gratitude to other professors and teachers: Dr. Bo Lei, Prof. Baojun Xu and Prof. Yang who gave me helpful supports and advices to my study. In addition, I would like to thank Mr. Chung, Dr. Tyler Leung and Dr. Cathy Lui for their technical support and help during my study.

Moreover, it is my great pleasure to give thanks to Lin Kaili, Peng Qiuxian, Zhang Fan, Xin Xiong, Zou Yukai, Du Bin, Liu Rui, Xu Chang, Liao Yanqing, Wu Benpei, Zhang Leimin, Wang Luyao, Zhao Boxin, Fu Xiuqiong and Liu Bin for their friendship, warm accompany and support.

Finally, I owe my deepest gratitude to my family for their understanding and endless support.
Table of Contents

DECLARATION ...................................................................................................................... I

ABSTRACT .......................................................................................................................... II

ACKNOWLEDGMENTS ....................................................................................................... V

TABLE OF CONTENTS ....................................................................................................... VI

LIST OF TABLES .................................................................................................................. VIII

LIST OF FIGURES .............................................................................................................. IX

LIST OF SYMBOLS ............................................................................................................ XII

LIST OF ABBREVIATIONS .................................................................................................. XIII

CHAPTER 1 GENERAL INTRODUCTION .............................................................................. 1

1.1 Background .................................................................................................................... 1

1.2 Research Objectives .................................................................................................... 13

1.3 Significance .................................................................................................................. 13

CHAPTER 2 MATERIALS AND METHODS ....................................................................... 15

2.1 Materials ...................................................................................................................... 15

2.2 Methods ....................................................................................................................... 16

CHAPTER 3 THE CYTOTOXIC EFFECT OF 2DG IN CANCER CELLS IS DUE TO THE DEPLETION OF GSH INDUCED BY AR/ARL ACTIVITY ........ 25

3.1 Introduction ................................................................................................................... 25

3.2 Objective ..................................................................................................................... 26

3.3 Results ......................................................................................................................... 26

3.4 Discussion .................................................................................................................... 62
3.5 Conclusion ........................................................................................................... 67

CHAPTER 4 BETTER SUBSTRATES FOR AR/ARL THAN 2DG ARE BETTER ANTI-CANCER AGENTS THAN 2DG ................................................................. 70

4.1 Introduction ........................................................................................................ 70
4.2 Objective ........................................................................................................... 70
4.3 Results ............................................................................................................... 71
4.4 Discussion ......................................................................................................... 102
4.5 Conclusion ........................................................................................................ 109

CHAPTER 5 SUMMARY AND FUTURE PROSPECTS ............................................. 110

5.1 Summary .......................................................................................................... 110
5.2 Future prospects .............................................................................................. 111

REFERENCES ........................................................................................................ 112

CONFIDENTIAL ..................................................................................................... 121

APPENDIX I ........................................................................................................... 122

APPENDIX II .......................................................................................................... 123

CURRICULUM VITAE ............................................................................................ 126
List of Tables

Table 2.1 Dilution rate of primary antibody and secondary antibody ..................21

Table 4.1 Acute mortality test by glyceraldehyde in mice..................................91
Table 4.2 The weight change after glyceraldehyde injection in mice......................91
Table 4.3 Acute mortality test by diacetyl in mice..............................................93
Table 4.4 The weight change after diacetyl injection in mice ................................93
List of Figures

Figure 1. 1 Structures of glucose and 2-deoxy-D-glucose ........................................... 2
Figure 1. 2 Warburg effect ............................................................................................... 3
Figure 1. 3 The mechanism of 2DG inhibit glycolysis ..................................................... 7
Figure 1. 4 Structure of 2FDG ......................................................................................... 8
Figure 1. 5 The hypothesis regards to mechanism of 2DG treat cancer cells .......... 11

Figure 3. 1 2DG cytotoxicity on HepG2 cells ................................................................. 28
Figure 3. 2 2DG cytotoxicity on SKOV3 cells ............................................................... 29
Figure 3. 3 2DG cytotoxicity on HCT116 cells ............................................................. 30
Figure 3. 4 2DG cytotoxicity on CaCo2 cells ............................................................... 31
Figure 3. 5 2DG cytotoxicity on SW480 cells ............................................................... 32
Figure 3. 6 2DG cytotoxicity on HT29 cells ................................................................... 33
Figure 3. 7 The relative levels of AR/β-actin and ARL/β-actin in six kinds of cells.... 35
Figure 3. 8 The influence of AR/ARL inhibitor on HepG2 cells ..................................... 37
Figure 3. 9 The influence of AR/ARL inhibitor on SKOV3 cells .................................. 38
Figure 3. 10 The influence of AR/ARL inhibitor on HCT116 cells .............................. 39
Figure 3. 11 The influence of AR/ARL inhibitor on CaCo2 cells ................................. 40
Figure 3. 12 The influence of MG-132 on HT29 and SW480 cells ............................. 42
Figure 3. 13 The influence of Bortezomib on HT29 and SW480 cells ...................... 43
Figure 3. 14 Survival rates of HepG2 cells when added 2DG together with AR/ARL inhibitor .................................................................................................................... 45
Figure 3. 15 Survival rates of SKOV3 cells when added 2DG together with AR/ARL inhibitor .................................................................................................................... 46
Figure 3. 16 Survival rates of HCT116 cells when added 2DG together with AR/ARL inhibitor .................................................................................................................... 48
Figure 3. 17 Survival rates of CaCo2 cells when added 2DG together with AR/ARL inhibitor .................................................................................................................... 49
Figure 3. 18 GSH level of HepG2 and SKOV3 cells when added 2DG together with AR/ARL inhibitor

Figure 3. 19 GSH level of HCT116 and CaCo2 cells when added 2DG together with AR/ARL inhibitor

Figure 3. 20 Survival rates of HT29 cells and SW480 cells when added 2DG together with MG-132

Figure 3. 21 Survival rates of HT29 cells and SW480 cells when added 2DG together with Bortezomib

Figure 3. 22 The expression of AR and ARL in HT29 cells induced by MG-132

Figure 3. 23 The expression of AR and ARL in SW480 cells induced by MG-132

Figure 3. 24 The expression of AR and ARL in HT29 cells induced by bortezomib

Figure 3. 25 The expression of AR and ARL in SW480 cells induced by bortezomib

Figure 3. 26 Molecular structures of tolrestat and fidarestat

Figure 3. 27 Molecular structures of MG-132 and bortezomib

Figure 4.1 The toxicity of glyceraldehyde on six cancer cells in 24 hours

Figure 4.2 The toxicity of glyceraldehyde on six cancer cells in 48 hours

Figure 4.3 The toxicity of diacetyl on six cancer cells in 24 hours

Figure 4.4 The toxicity of diacetyl on six cancer cells in 48 hours

Figure 4.5 Enzyme activity assay of glyceraldehyde, diacetyl and 2DG

Figure 4.6 Cytotoxicity of various AKR substrates at concentration of 5mM/2.5mM for 48 hours

Figure 4.7 Cytotoxicity of various AKR substrates at concentration of 5mM/2.5mM for 24 hours

Figure 4.8 GSH level on cancer cells treated by glyceraldehyde and diacetyl

Figure 4.9 BSO enhanced the cytotoxicity of glyceraldehyde on cancer cells

Figure 4.10 BSO enhanced the cytotoxicity of diacetyl on cancer cells

Figure 4.11 NAC protected cancer cells against the cytotoxicity of glyceraldehyde

Figure 4.12 NAC protect cancer cells against the cytotoxicity of diacetyl

Figure 4.13 The tumor volume of glyceraldehyde (500mg/kg) in tumor xenografts
Figure 4.14 The body weight of glyceraldehyde (500mg/kg) in tumor xenografts study

Figure 4.15 The tumor weight of glyceraldehyde (500mg/kg) in tumor xenografts study

Figure 4.16 The tumor volume of diacetyl (80mg/kg) in tumor xenografts study

Figure 4.17 The body weight after diacetyl (80mg/kg) in tumor xenografts study

Figure 4.18 The tumor weight after diacetyl (80mg/kg) in tumor xenografts study

Figure 4.19 Molecular structures of glyceraldehyde and diacetyl
LIST OF SYMBOLS

μg: microgram

mg: milligram

g: gram

kg: kilogram

μl: microliter

ml: milliliter

L: liter

mg/kg: milligram per kilogram

g/L: gram per liter

mg/ml: milligram per milliliter

g/mol: gram per mol

μg/ml: microgram per milliliter

μmol/L: micromole per liter

mmol/L: millimole per liter

mmol/ml: millimole per milliliter

rpm: revolutions per minute
LIST OF ABBREVIATIONS

2DG: 2-deoxy-D-glucose

2FDG: 2-fluoro-deoxy-glucose

ATP: Adenosine Triphosphate

AR: Aldose reductase (AKR1B1)

ARL: Aldose reductase like (AKR1B10)

AKRs: Aldo-keto reductase family

ARI: Aldose reductase inhibitor

ATCC: American Type Culture Collection

APS: Ammonium persulfate

BSO: L-buthionine-[S,R]-sulfoximine

BW: Body weight

CAS: Chinese Academy of Sciences

DMEM: Dulbecco’s Modified Eagle Medium

DMSO: Dimethylsulfoxide

EB: Electrophoresis buffer

FBS: Fetal bovine serum

GSH: Reduced glutathione

GSSG: Oxidized glutathione

GLUT: Facilitative glucose transporter

GR: Glutathione reductase

LD50: Median lethal dose
MTD: Maximum tolerated dose

mRNA: Messenger RNA

NADPH: Nicotinamide adenine dinucleotide phosphate

NADP+: Oxidized form of nicotinamide adenine dinucleotide phosphate

NADH: Nicotinamide adenine dinucleotide hydrogen

NAD+: Oxidized form of nicotinamide Adenine Dinucleotide Hydrogen

NAC: N-acetyl-cysteine

NCS: Newborn calf serum

NRF2: Nuclear factor erythroid 2-related factor 2

PSN: Penicillin, Streptomycin, Neomycin

PBS: Phosphate buffered saline

ROS: Reactive oxygen species

SDH: Sorbitol dehydrogenase

SDS: Sodium dodecyl sulfonate

TBS: Tris buffer saline

TBST: Tris buffer saline with tween-20

TEMED: Tetramethylethylenediamine
Chapter 1 General Introduction

1.1 Background

1.1.1 2-deoxy-D-glucose

2-deoxy-D-glucose (2DG) is a non-metabolized glucose analog in which the 2-hydroxyl group is replaced by a hydrogen atom (Figure 1.1). It cannot participate in glycolysis, and thus it induces a state of glucose deprivation by competing with glucose transporter for uptake and metabolism (Aft, Zhang, & Gius, 2002).

1.1.2 Warburg effect

In 1920, Otto Warburg observed that tumor cells utilized non-oxidative breakdown of glucose called glycolysis for producing ATP rather than oxidative phosphorylation, even in the present of sufficient oxygen. This phenomenon in cancer cells is called Warburg effect. This is different with healthy cells that primarily generate ATP through oxidative phosphorylation in the mitochondria which generates far more ATP per molecule of glucose than glycolysis. Warburg found that cancer cells have higher ratio of glycolytic activity over oxidative phosphorylation than normal cells (Figure 1.2).
2-deoxy-D-glucose (2DG) is a non-metabolized glucose analog in which the 2-hydroxyl group is replaced by hydrogen group.
Figure 1. 2 Warburg effect

The Warburg effect is the observation that most cancer cells predominantly produce energy by a high rate of glycolysis followed by lactate fermentation, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells.
Tumor cells undergo very high rates of glycolysis even in the aerobic conditions. As a result, cancer cells are more sensitive to the inhibition of glycolysis. In early research, 2DG inhibited the growth of several kinds of tumors in rodent model, which led to testing 2DG as an anticancer agent in cancer patients (Cay, Radnell, Jeppsson, Ahren, & Bengmark, 1992; Ball, Wick, & Sanders, 1957). However, 2DG in clinical trials were unsuccessful because it was ineffective and also there were some side effects (Ahrén B., Bood M, 1987; Karlsson, & Ahren, 1987). In fact, under the sufficient oxygen and nutrients, glycolytic inhibition could only slow down the cancer develop instead of killing the cancer cells. It was suggested that some other alternative energy sources were utilized in cancer cells, such as the breakdown of proteins and fats.

1.1.3 2DG used in clinical cancer therapy

Tumor environment is different from normal tissue environment. Due to the rapid growth that outpaces vascularization larger tumors contain hypoxic regions. This region of tumor is usually resistant to standard chemotherapeutic agents (Harrison, Chadha, Hill, Hu, & Shasha, 2002). On the other hand, tumor cells growing under hypoxia were shown to be sensitive to glycolysis inhibition because these tumor cells mainly relied on glycolysis for the generation of ATP for their energy needs (Liu, Savaraj, Priebe, & Lampidis, 2002; Maher, Savaraj, Priebe, Liu & Lampidis, 2005).

This phenomenon of different glucose metabolism between tumor and normal tissue gives the opportunities for tumor therapy. Cancer cells need more glucose than
normal cells and therefore try to import more glucose. Since 2DG is also imported by glucose transporters, 2DG accumulates in cancer cells more than other cells. 2DG taken up by the glucose transporters inhibits phosphor-hexose isomerase, which can convert phosphor-glucose to phosphor-fructose. Since 2DG cannot undergo further glycolysis, these cells will die because glycolysis is blocked by 2DG (LaManna, & Lust, 1997; Boros, et al., 1998) (Fig 1.3). This has been shown by previous reports (Maher, Krishan, & Lampidis, 2004).

The cytotoxicity of 2DG is not as severe as some anticancer drugs such as doxorubicin or cisplatin. However, its specificity in killing cancer cells makes it a good candidate to enhance the efficacy of other cancer therapies. It has been shown to have synergistic effect in enhancing the killing of breast cancer cells by 5-fluorouracil, doxorubicin, cisplatin, cyclophosphamide, and herceptin (Chung, Ho, Lam, & Chung, 2003). 2DG enhances apoptosis of melanoma cells induced by TNF-related apoptosis ligands (Scott, & Viola, 1998). 2DG was also tested in clinical trials to enhance radiation therapy. It has been shown that 2DG induced sensitization to radiation in breast cancer cells appeared to be dependent on the presence of functional p53 (Islamian, Aghaee, Farajollahi, Baradaran, & Fazel, 2015). However, the mechanism of 2DG’s enhancement of radiation therapy is still not completely clear.

2DG was shown to be effective at inhibiting glucose metabolism without any toxicity in experiment animals (Mohanti, et al., 1996). A recent clinical trial also showed that 2DG administered to human once weekly was tolerated up to a dose of 200 mg/kg body weight (Aft, Zhang, & Gius, 2002). Thus, 2DG can be used safely to
induce a state of glucose depletion to enhance cancer therapies. However, the observed cardiac side-effects and the fact that a majority of patients' cancer continue to progress casts doubt on the feasibility of this drug for further clinical use.

1.1.4 Novel mechanism of 2DG mediates cancer treatment

It is not completely clear how 2DG inhibits cancer cells growth. The fact that glycolysis is inhibited by 2DG seems not to be sufficient to explain why 2DG treated cells stop growing. Previous report showed that 2DG only inhibited glycolysis by 15-40%, not sufficient to kill cancer cells (Kurtoglu, Gao, & Shang, et al., 2007). Further, 2-fluoro-deoxy-glucose (2FDG), a more potent inhibitor of glycolysis, was found to be not effective in cancer treatment (Kelloff et al., 2005) (Figure 1.4).

These two observations indicate that the mechanism of 2DG mediate cancer treatment is not glucose deprivation or inhibit glycolysis. Since 2DG’s cytotoxicity is specific to cancer cells, but it is only marginally effective as an anticancer drug, we would like to find out the mechanism of its cancer-specific cytotoxicity so that better anticancer drugs can be developed.

Previous report showed that reactive oxygen species (ROS) was increased in cancer cells after treatment with 2DG (Shutt, O’Dorisio, Aykin-Burns, & Spitz, 2010). This phenomenon indicated that cytotoxic effect of 2DG might be due to increase ROS.
2DG can inhibit phosphor-hexose isomerase which converts phosphor-glucose to phosphor-fructose; as a result, 2DG inhibits glycolysis.
Figure 1. 4 Structure of 2FDG

2FDG which was a more potent inhibitor of glycolysis, however, it is not effective in cancer treatment.
ROS, which formed as a natural byproduct of the normal metabolism, is a group of chemically reactive molecules that contain oxygen free radicals (Devasagayam, et al., 2004). ROS is implicated in a variety of inflammatory responses, which can induce cells damage including cancer cells. Generally, the harmful effects of ROS in cells have several aspects, including DNA damages, oxidation of polyunsaturated fatty acid in lipids, amino acids oxidation in proteins, and damages to cell structures (Hwang, Hwang, & Song, 2016).

1.1.5 The hypothesis on the mechanism of 2DG cytotoxicity

We can start explaining the hypothesis with two metabolic pathways, polyol pathway and glutathione synthesis pathway. Polyol pathway, which also called sorbitol-aldose reductase pathway, is important in human glucose metabolism and has been implicated in various diabetic complications (Mima, 2016; Soltesova, Ballekova, Gajdosikova, Gajdosik, & Stefek, 2015). The polyol pathway was first identified by Hers, who demonstrated that blood glucose can be turned into fructose by this pathway (Hers, & Hue, 1983).

In the polyol pathway, aldose reductase (AR) enzyme and aldose reductase like (ARL) enzyme reduce glucose to sorbitol. In the process, they oxidize NADPH (Nicotinamide adenine dinucleotide phosphate) to NADP+. Then sorbitol is oxidized by sorbitol dehydrogenase to fructose, which converts NADH (Nicotinamide adenine dinucleotide hydrogen) from NAD+. Another important pathway is the regeneration of GSH (reduced glutathione) from GSSH (oxidized glutathione). In this pathway, GSSG
is reduced to GSH by GR (glutathione reductase). This reaction also needs NADPH as a co-factor.

Both of these two reactions are competing for NADPH as their co-factor. 2DG can also be reduced by AR and ARL (ref). When 2DG is added to the cancer cells, more NADPH will be consumed in the polyol pathway, and consequently less NADPH will be available for the GSH synthesis. As the result, the level of GSH will be decreased. In healthy tissue, over 90% of glutathione is exists in GSH and less than 10% exists in GSSG (Halprin & Ohkawara, 1967). GSH is the most important endogenous antioxidant, participating in neutralizing free radicals and reactive oxygen compounds. GSH can prevent cell damages caused by ROS. The decreased GSH level will induce the level of ROS increased, contributing to cell death.

The adverse effects of the polyol pathway were demonstrated in diabetic animals. When the concentration of intracellular glucose is too high, AR reduces glucose to sorbitol, which is then turned to fructose by sorbitol dehydrogenase. Under hyperglycemic conditions, the polyol pathway increases intracellular oxidative stress by decreasing the concentration of GSH (Lee & Chung, 1999; Brownlee, 2005). Since cancer cells often overexpress AR and/or ARL, we proposed that the cytotoxic effects of 2DG in cancer cells are due to the depletion of GSH as a result of reduction of 2DG by AR/ARL (Figure 1.5).
Figure 1. The proposed mechanism of 2DG cytotoxicity

In the presence of 2DG, more NADPH will be consumed by AR/ARL, and consequently less NADPH will be available for the regeneration of GSH. The decreased GSH level will increase the level of ROS, contributing to cell death.
1.1.6 Aldose reductase (AR) and aldose reductase like (ARL)

Both AR (also named AKR1B1) and ARL (also named AKR1B10) are members of the aldo-keto reductase (AKRs) family of NADPH dependent enzymes (Jez, & Penning, 2001; Kaneko, et al., 2005; Ramasamy, & Goldberg, 2010). The molecular weight of the AKRs ranges from 34-37 kDa. About 115 members have been identified in the super family (Grimshaw, Bohren, Lai, & Gabbay, 1995). Both AR and ARL consist of 316 amino, and 71% their amino acid sequence is identical to each other. AR and ARL have similar enzymatic properties and substrate specificity due to the similarity of their structure including the active sites. AR and ARL differ from each other in their pH optimum, salt requirement, and the kinetics in reducing some of these substrates. The AR and ARL are expressed in different tissues, suggesting that they may have different physiological functions. (Cao, D., Fan, S.T., & Chung, S.S., 1998)

Due to its adverse effects when reducing glucose, AR is an attractive target for the treatment of diabetic complications. It was reported that AR inhibitor (ARI) fidarestat treatment significantly altered the progression of neuropathy in patients with diabetes. Subjective symptoms were significantly improved in fidarestat group versus the placebo group (Hotta, et al., 2001). Another kind of ARI, tolrestat, was also used to stop the progression of diabetic autonomic neuropathy (Didangelos, et al., 1998).

There are many kinds of substrates for AR/ARL, and glucose is one of the least efficient substrates for these two enzymes. 2DG had been shown to serve as a substrate for AR (Yabe-Nishimura, 1998). It is a better substrate for AR than glucose, but not as good as other substrates, such as glyceraldehyde and diacetyl. Our hypothesis predicts
that glyceraldehyde and diacetyl would be more effective in killing cancer cells that overexpress AR/ARL than 2DG.

1.2 Research Objectives

There are two main objectives in this research project. One is to identify cancer cells that are sensitive to 2DG and also cancer cells that are resistant to 2DG, and use these cells to determine the relationship between sensitivity to 2DG and the express level of AR/ARL. With the 2DG sensitive cells and 2DG non-sensitive cells, we can inhibit AR/ARL activities, increase AR/ARL to test our hypothesis that the cytotoxicity of 2DG is due to GSH depletion as a consequence of the reduction of 2DG by AR or ARL.

Another objective of this project is to determine if better substrates for AR/ARL would have better anti-cancer effect than 2DG. If the hypothesis is proven correct, this would lead to the development of a new class of anticancer drugs.

1.3 Significance

This research project is significant and relevant because cancer is the most prevalent disease world-wide, with a high mortality rate. 2DG, a non-metabolizable glucose analog, is currently in clinical trials to enhance the efficacy of radiotherapy and chemotherapy of various cancers. It is thought to kill cancer cells by inducing glucose deprivation state. However, we proposed that the cytotoxic effect of 2DG is due to the depletion of GSH rather than inhibition of glycolysis. Our present study is to
verify our hypothesis. This proposed mechanism better explains the cytotoxicity effect of 2DG in cancer cells. More importantly, if the hypothesis is proven correct, it will lead to the development of more effective anticancer drugs.
Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Cells lines

The SKOV3, CaCo2 and HCT116 cell lines were purchased from the Shanghai Cells Bank, Chinese Academy of Sciences (CAS); the HepG2 and SW480 cell lines were obtained from Prof. Li Guofeng (School of Pharmacy, Southern Medical University) as a gift; and the HT29 cell line was a gift from Dr. Shi Jue (Faculty of science, Hong Kong Baptist University).

2.1.2 Experimental animals

ICR mice (18-22g, half male and half female) were acquired from Laboratory Animal Services Centre in The Chinese University of Hong Kong; BALB/C nude mice (Six-week-old, male) were purchased from the Laboratory Animal Unit in The University of Hong Kong. The handling of animals and procedures involving in the use of animals were approved in accordance with the Animals Ordinance, Hong Kong Department of Health, Hong Kong SAR. All efforts were made to ensure that both animal numbers and sufferings were minimized in the experiment.

2.1.3 Experimental drugs

2-deoxy-D-glucose (2DG, crystalline, >99% (GC), Cat.No.: D6134); glyceraldehyde (Cat.No.: G5001) and diacetyl (Cat.No.: B85307) were purchased from Sigma.
2.1.4 Antibodies

Monoclonal Anti-Mouse β-actin antibody (Cat.No.: A5316) was purchased from Sigma; Rabbit Anti-AKR1B10 (ARL) antibodies (Cat.No.: ab139685) was purchased from Abcom; Rabbit Anti-AKR1B1(AR) antibodies was a gift from Dr. Deliang Cao of Southern Illinois Medical School; HRP-Goat Anti-Mouse IgG (H+L) (Cat.No.: 626520) and HRP-Goat Anti-Rabbit IgG (H+L) (Cat.No.: 656120) were purchased from Invitrogen.

2.2 Methods

2.2.1 Cells culture

HepG2 cells, SKOV3 cells, HCT116 cells and CaCo2 cancer cells were cultured in DMEM complete medium (10% FBS, 1% PSN); SW480 in 1640 complete medium (10% FBS, 1% PSN); HT29 in McCoy’s 5A complete medium (10% FBS, 1% PSN).

Cells culture medium was warmed to 37°C before used. 6 ml of fresh complete medium was added to the 25 cm² culture flask and 15 ml was added to the 75 cm² culture flask and then incubated at 37°C with 5% CO₂ and 95% constant humidity. The medium was changed every two days followed by rinsing with sterile PBS. Possible contamination with bacteria, yeast and fungi was monitored by inverted phase contrast microscope (Drexler, & Uphoff, 2002). The logarithmic growth phase cells were used for experiment.

Subculture: The spent medium was removed gently, and cells were washed twice
with PBS and then trypsin/EDTA was added. The harvested cells were collected into centrifuge tubes and centrifuged for 5 minutes at 1000 rpm. The medium was discarded and cells re-suspended into flasks or plate. The ratio of subculture was 1:3~1:5. Cells were counted using the coulter counter. The average cells number in 4 areas (each area which contains 16 smaller squares) was calculated.

\[
\text{Cell density} = \frac{N}{4} \times F \times 10^4 / \text{ml} \quad (N: \text{Total number of 4 areas}; F: \text{Dilution factor})
\]

**Cell cryopreservation:** The harvested cells were suspended in freezing medium (60% complete medium, 30% FBS, 10% DMSO; freshly prepared) and the cells density was adjusted to 1~10×10⁶ cells/ml. 1 ml cells in freezing medium was added to each freezing vial. The vials were gradient freezing with 4°C for 30 minutes, -20°C for 1 hour, overnight at -80°C, and then transferred to liquid nitrogen freezer.

**Thawing:** A vial was removed from liquid nitrogen and placed into 37°C water bath as soon as possible and then centrifuged at 1000 rpm for 5 minutes. The medium was removed and the cells were re-suspended. Cells were poured into flask and added warm complete medium. Medium was changed after 24 hours.

**2.2.2 Detection of cell survival rate by MTT experiment**

Cells were harvested and adjusted to 5×10⁴ cells/ml. 200μl cells suspension was plated in each well in a 96 wells plate (1×10⁴ cells/well) and incubated at 37°C, 5%CO₂ overnight. The no drug-treat cells group was served as NC group. Each group had 5
wells. The medium was removed and 150μl MTT working solution (5mg/ml, 1:10 dilution) was added into each well. The plate was then covered with tinfoil and incubated at 37℃ for 4 hours. The medium was then removed and 150μl DMSO was added to dissolve the precipitate. The plate was shaken for 30 min. OD value was measured at 540 nm by the plate reader. The survival ratio of cancer cells was calculated with the equation:

\[
\text{Cells survival ratio} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{NC}} - \text{OD}_{\text{blank}})} \times 100\%
\]

\(\text{OD}_{\text{blank}}\): OD value of no-cells well; \(\text{OD}_{\text{sample}}\): OD value of cells induced by drug well; \(\text{OD}_{\text{NC}}\): OD value of no drug-treat well.

The survival rate of cancer cells were tested by MTT experiment after different concentrations of 2DG was added for 24, 48 or 72 hours. The survival rates of cancer cells induced by 2DG in the presence of AR/ARL inhibitor or AR/ARL inducer were measured by MTT experiment. The concentrations of AR/ARL inhibitor or inducer used in the experiment did not affect cell survival. In this test, 2DG was added to cancer cells after pretreated with AR/ARL inhibitor or inducer for 24 hours, and then incubated at 37℃, 5%CO₂ for additional 48 hours. On the other hand, the survival rate of cancer cells was tested by MTT experiment after different concentrations of better AR/ARL substrates were added for 24 or 48 hours.

2.2.3 Determination of the levels of AR/ARL protein by Western blot analysis

2.2.3.1 Protein sample preparation

Cells were seeded on the 6 well plates. When cells were grown to 80~90%
confluent and still in logarithmic growth, cells were harvested for protein extraction. The plates were on ice and the medium removed and cells were washed by cold PBS three times. 20μl lysis buffer was then added to each well and kept on ice for 30 minutes. Cells were scraped off the well by a cells scraper. The cells lysate was collected and then centrifuged at 14000g/min at 4°C for 30 minutes. Equal volume of 2× sample buffer was added and then heated at 95°C for 5 minutes. Extract samples were stored at -20°C.

2.2.3.2 Protein concentration determination

5μl sample (18μl water + 2μl extracts) was added in a well of 96 well plates. Each sample had three parallel trials. Protein analytical reagents were added to the well according to the directions. The plates were wrapped with aluminum foil and stand at room temperature for 30 minutes. The absorbance at 750 nm was determined by microplate reader. The concentration of protein sample was calculated by the following equation:

\[
\text{Mass} \ (\mu g/ml) = \left[ \frac{\text{Absorbance} - 0.0865}{0.1555} \right] \times 10 \times 1000
\]

2.2.3.3 Electrophoresis and electro-blotting

Equal amount of protein sample was loaded (20 μg) and molecular weight marker was added in 10% SDS-PAGE gel. The electrophoresis was run at room temperature at 40V, 50W and 130mA for 30 minutes and then the voltage was changed to 75V until the dye reached the bottom of the gel (Keep voltage constant).
The transfer buffer was freshly prepared. The gel sandwich was prepared as the following sequence: (-) Filter paper, Gel, PVDF membrane, Filter paper (+). The gel sandwich was put into the tank and protein was transferred to the membrane at room temperature at 15V, 50W and 110mA (voltage constant) overnight with the buffer being stirred by the magnetic stirrer bar.

2.2.3.4 Detection of protein bands by antibodies

The membrane was collected and washed with 1× TBST for 10 minutes. The membrane was incubated in blocking agent (5% non-fat milk) for 1 hour at room temperature or overnight at 4°C. Membrane was incubated with appropriate dilutions of primary antibody in 2% milk for 2 hours at room temperature or overnight at 4°C. The filter was rinsed with 1× TBST 3 times for 5 minutes each time and incubated with second antibody in 2% milk for 1 hour. (Table 2.1)

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibody</td>
<td></td>
</tr>
<tr>
<td>Anti-AKR1B1(AR)</td>
<td>1:3000</td>
</tr>
<tr>
<td>Anti-AKR1B10(ARL)</td>
<td>1:3000</td>
</tr>
<tr>
<td>Anti-β-actin</td>
<td>1:5000</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td></td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>1:5000</td>
</tr>
<tr>
<td>Goat anti-rabbit</td>
<td>1:5000</td>
</tr>
</tbody>
</table>
The filter was rinsed with 1X TBST 3 times for 5 minutes each time. The chemiluminescent substrate was added onto the membrane for 1 minute and then the fluorescent image was captured in X-ray film. 4 ml stripping buffer was added to the membrane placed on a shaker for 15 minutes. After washing with TBST for 20 minutes, the membrane could be reused.

2.2.4 Assays for GSH level

Cells were pretreated with AR/ARL inhibitors (40μM) for 24 hours, followed by the addition of 2DG (20mM) and incubated for additional 48 hours. The cells were then harvested for GSH level analysis. The cells were disrupted by ultrasonicator on ice and then centrifuged with 3500 rpm at 4°C for 10 mins. The supernatant was used for further GSH assay by GSH assay kit (Cat.No.: 20150120, NJJC Bioengineering Institute). GSH analytical reagents were added according to the directions and then incubated at room temperature for 5 mins. OD value was measured at 400 nm by the plate reader. The level of GSH in cells lysates were measured by using the standard GSH (20μmol/L) and related to protein concentrations. The assays of GSH levels were done at least three times. The GSH level was calculated with the equation:

\[
\text{GSH (nmol/mg)} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}})} \times 20\mu\text{mol/L} \times \frac{F}{P} \times 1000
\]

(F: Dilution factor; P: Protein concentrations)

2.2.5 Determination of catalytic efficiency of AR/ARL substrates by enzyme activity assay
Enzyme activity assay was detected at 30°C for 30 min in 1 ml of the reaction mixture containing 135mM sodium phosphate buffer (pH 6.2 for AR or pH 7.0 for ARL), 0.2mM NADPH, 0.3M ammonium sulfate, 2μg purified AR/ARL protein and 20mM AR/ARL substrates (glyceraldehyde, diacetyl and 2DG). Protein-free were detected as blank control. The amount of NADPH was tested at 340nm by spectrophotometer. Enzyme activity was calculated as the decreased amount of NADPH oxidized/min/mg protein.

2.2.6 The AR/ARL substrates’ cytotoxicity was associated with intracellular GSH levels

In our hypothesis, GSH play a very important role in AR/ARL substrate toxicity on cancer cells. To examine this, the cytotoxicity of glyceraldehyde and diacetyl on cancer cells with changing the level of GSH were detected.

BSO was used to decrease the level of GSH in cancer cells, and NAC was used to increase the level of GSH in cancer cells. Firstly, the survival rates of cancer cells were tested after treated with diacetyl (Dia: 1.25mM and 0.625mM) or glyceraldehyde (Gly: 1.25mM and 0.625mM) in the absence or presence of BSO (0.3mM) for 48h. Secondly, the survival rates of cancer cells were tested after treated with diacetyl (Dia: 2.50mM and 1.20mM) or glyceraldehyde (Gly: 2.50mM and 1.25mM) in the absence or presence of NAC (1mM) for 48h. The survival rates were tested by MTT experiment.
2.2.7 Determination of LD50 of glyceraldehyde and diacetyl in acute toxicity test

Male and female ICR mice were housed separately in 4 mice per cage in a well-ventilated room with 12 hours cycle of day and night light conditions and the temperature in room was maintained at around 25°C. Diacetyl and glyceraldehyde were dissolved with saline water. Each group had four male and four female ICR mice and received the treatment of a single dose of glyceraldehyde or diacetyl by intravenous injection according to the body weight of each animal. Mice were individually observed for the first 30min after injection, regularly observed for the first 24 hours, and daily for total 14 days for the mortality or toxicity. The number of dead mice was recorded and used in the calculation of LD50 value. The surviving mice were weighted regularly and euthanized with diethyl-ether at the end of test.

2.2.8 Determination of anti-cancer effect of glyceraldehyde and diacetyl in xenograft tumor model

Six-week-old male BALB/C nude mice were allowed to acclimate to the laboratory conditions for 1 week before cancer cells injection. Human HepG2 tumor xenografts were established by injecting $5\times10^6$ HepG2 cells/mice in the left flanks of nude mice. Treatment was initiated when the tumor grew to about 200±100 mm$^3$. Each treatment group had six mice. Mice were randomized for different experimental groups and received the daily tail vein injection for 3 weeks (6 days a week): 500mg/kg body weight of glyceraldehyde; normal saline for the control group. Mice were weighted and tumors volumes were measured every 3 days by caliper. Tumor volumes were
calculated with the formula: \( \text{area} = \frac{\text{length} \times \text{width}^2}{2} \). Animals were humanely killed at the end of experiments and tumors were weighted. The anti-cancer effect of glyceraldehyde and diacetyl were tested separately. The method of diacetyl test was same as glyceraldehyde and the dose of diacetyl used was 80 mg/kg.

2.2.9 Statistics

Data were expressed as mean \( \pm \) SD. Western blot result was analyzed with ImageJ software for Windows. Statistical analysis was performed by SPSS 13.0 system software. One-way ANOVA was used to determine P value (LSD test for equal variances assumed; Dunnett’s T3 test for equal variances not assumed). P<0.05 was accepted as statistically significant.
Chapter 3 The cytotoxic effect of 2DG in cancer cells is due to the depletion of GSH induced by AR/ARL activity

3.1 Introduction

Cellular sensitive to 2DG varies in different cancer cell lines. According to our hypothesis, cells that overexpress AR and/or ARL would be more sensitive to 2DG. HepG2, SKOV3, HCT116, CaCo2, SW480 and HT29 cells were tested to see which are 2DG sensitive and which are 2DG non-sensitive. The relationship between the expression of AR/ARL protein and 2DG sensitivity in these cancer cells was determined by several approaches.

Besides determining whether there is a correlation between sensitivity to 2DG and overexpression of AR/ARL, we want to determine if inhibition of AR/ARL activities in cells that have high levels of these enzymes would protect these cells against 2DG toxicity. Two kinds of inhibitors, fidarestat and tolrestat, were used to inhibit the AR/ARL activity. AR/ARL inhibitors would protect 2DG sensitive cancer cells against 2DG toxicity if our hypothesis is correct. At the same time, inhibition of AR/ARL would restore the drop of GSH induced by 2DG as we proposed that cytotoxicity of 2DG is due the depletion of GSH.

On the other hand, 2DG non-sensitive cells would have relatively low level of AR/ARL proteins. We want to determine if these cells would be more sensitive to 2DG when their AR/ARL protein levels are increased. Two kinds of AR/ARL inducer, MG-132 and bortezomib (PS-431) will be used to increase the expression of AR/ARL
protein. If our hypothesis is correct, AR/ARL inducer would make 2DG non-sensitive cancer cells more sensitive to 2DG. These experiments could better explain that the cytotoxicity effect of 2DG in cancer cells is due to the activity of AR and/or ARL which are over-expressed in some kinds of the cancer cells.

3.2 Objective

The objective of the experiments described in this chapter is to determine if the cytotoxic effect of 2DG in cancer cells is due to depletion of GSH as a consequence of the reduction of 2DG by the enzymatic activities of AR/ARL.

3.3 Results

3.3.1 The sensitivity of HepG2, SKOV3, HCT116, CaCo2, SW480 and HT29 cancer cells to 2DG.

In the present experiment, different concentration of 2DG (40, 20, 10, 5 and 2.5 mmol/L) were added to cultures of HepG2, SKOV3, HCT116, CaCo2, SW480 and HT29 cancer cells. The cell survival rates after 24, 48 and 72 hours treatment were determined by MTT assays.

From the result of MTT experiment, the survival rate of HepG2 cells was found to be decreasing rapidly when increasing concentrations of 2DG were added. When concentration of 2DG was 40mmol/ml, HepG2 cells survival rate decreased to about 25%. For SKOV3 cells, the survival rate also dramatically decreased when 2DG were
added. HepG2 cells and SKOV3 cells were considered as 2DG sensitive cancer cells. (Figure 3.1, Figure 3.2)

Similarly, the survival rate of HCT116 cells and CaCo2 cells also rapidly decreased when increasing concentrations of 2DG were added. HCT116 cells survival rate was dramatically decreased to about 25% when added with 40mmol/ml 2DG for 72 hours. In CaCo2 cells, the survival rate also dramatically decreased to about 30% when added with 40mmol/ml 2DG for 72 hours. HCT116 cells and CaCo2 cells were also considered as 2DG sensitive cancer cells. (Figure 3.3, Figure 3.4)

However, the survival rates of SW480 cells and HT29 cells decreased modestly after added different concentrations of 2DG, compared to other cancer cells. The survival rates of SW480 and HT29 cells remained at approximately 80% when added with 40mmol/ml of 2DG for 72 hours. Therefore, SW480 cells and HT29 cells were considered as 2DG non-sensitive cancer cells. (Figure 3.5, Figure 3.6)

To summarize, HepG2 cells, SKOV3 cells, HCT116 cells and CaCo2 cells were considered as 2DG sensitive cancer cells, SW480 cells and HT29 cells were considered as 2DG non-sensitive cancer cells.
Figure 3. 1 2DG cytotoxicity on HepG2 cells

The survival rate significantly decreased when 2DG was added. The survival rate of HepG2 cells dramatically decreased to about 25% when added 40mmol/L of 2DG for 72 hours. HepG2 cells were considered as 2DG sensitive cancer cells. Compared with NC group, **P<0.01.
Figure 3. 2DG cytotoxicity on SKOV3 cells

The survival rate significantly decreased when 2DG was added. The survival rate of SKOV3 cells dramatically decreased to about 40% when added 40mmol/L 2DG for 72 hours. SKOV3 cells were considered as 2DG sensitive cancer cells. Compared with NC group, **P<0.01.
Figure 3. 3 2DG cytotoxicity on HCT116 cells

The survival rate significantly decreased when 2DG was added. The survival rate of HCT116 cells dramatically decreased to about 25% when added 40mmol/L 2DG for 72 hours. HCT116 cells were considered as 2DG sensitive cancer cells. Compared with NC group, **P<0.01.
The survival rate significantly decreased when 2DG was added. The survival rate of CaCo2 cells dramatically decreased to about 30% when added 40mmol/L 2DG for 72 hours. CaCo2 cells were considered as 2DG sensitive cancer cells. Compared with NC group, **P<0.01.
The survival rate of SW480 cells decreased modestly, much less when compared with HepG2, SKOV3, HCT116 and CaCo2 cells. The survival rate of SW480 cells remained at about 80% when added 40mmol/L 2DG for 72 hours. SW480 cells were considered as 2DG non-sensitive cancer cells. Compared with NC group, *P<0.05; **P<0.01.
Figure 3. 6 2DG cytotoxicity on HT29 cells

The survival rate of HT29 decreased modestly, much less when compared with HepG2, SKOV3, HCT116 and CaCo2 cells. The survival rate of HT29 remained at about 80% when added 40mmol/L 2DG for 72 hours. HT29 cells were considered as 2DG non-sensitive cancer cells. Compared with NC group, *P<0.05; **P<0.01.
3.3.2 Detecting the expression of AR/ARL in cancer cells

Western blot experiment was done to determine the expression levels of AR/ARL in HepG2, SKOV3, HCT116, CaCo2, SW480 and HT29 cancer cells.

According to the result of the western blot experiment, the protein band of β-actin was almost the same in all six cell lines, and therefore it was used as a reference protein band. The levels of AR and ARL proteins were relatively high in both HepG2 cells and SKOV3 cells. HCT116 and CaCo2 cancer cells only over-expressed AR protein. On the other hand, both AR and ARL protein bands were low in HT29 cells, AR and ARL protein bands were almost invisible in SW480 cells. (Figure 3.7)

From the western blot results, HepG2 and SKOV3, which were 2DG sensitive, over-expressed both AR and ARL proteins; HCT116 and CaCo2, which were 2DG sensitive, only over-expressed AR protein. However, SW480 and HT29, which were 2DG non-sensitive, expressed low levels of both AR and ARL proteins. These results indicate that cells that were sensitive to 2DG expressed higher levels of AR/ARL than cells that were not sensitive to 2DG.
The expression of AR and ARL proteins were relatively high in both HepG2 cells and SKOV3 cells. HCT116 and CaCo2 cells only over-expressed AR protein. However, the levels of both AR and ARL proteins were very low in HT29 cells, AR and ARL protein bands were almost invisible in SW480 cells. (A) AR/ARL band of molecular weight ~ 36kDa and the β-actin band of molecular weight ~ 42kDa. (B) The histograms show the optical density of the bands after measurement by using the software (ImageJ).
3.3.3 The effects of AR/ARL inhibitors on HepG2 and SKOV3 cells

MTT experiments were done to determine the survival rate of HepG2, SKOV3, HCT116 and CaCo2 cancer cells in the presence of different concentrations of AR/ARL inhibitors, fidarestat and tolrestat.

The results show that the survival rates of HepG2 cells and SKOV3 cells were over 90% when the concentration of fidarestat and tolrestat was below 50μmol/L (Figure 3.8, Figure 3.9); the survival rates of HCT116 cells and CaCo2 cells were also over 90% when the concentration of fidarestat and tolrestat was below 50μmol/L (Figure 3.10, Figure 3.11).

In conclusion, fidarestat and tolrestat did not affect the survival rate of HepG2 cells, SKOV3 cells, HCT116 cells and CaCo2 cells when the concentration these two inhibitors was no higher than 50μmol/L.

According to the results of these experiments, the concentrations of fidarestat and tolrestat used in the following experiments were in the range of 40, 20 and 10μmol/L.
Figure 3. 8 The influence of AR/ARL inhibitor on HepG2 cells

The cells survival rate decreased to below 90% when the concentration of fidarestat and tolrestat reached 100μmol/L. Fidarestat and tolrestat had no effect on the survival rate of HepG2 when the concentration was no higher than 50μmol/L. Compared with NC group, *P<0.05; **P<0.01.
Figure 3.9 The influence of AR/ARL inhibitor on SKOV3 cells

The survival rate of SKOV3 cells decreased to below 90% when the concentration of fidarestat and tolrestat reached 100μmol/L. Fidarestat and tolrestat had no effect on the survival rate of SKOV3 cells when the concentration was no higher than 50μmol/L. Compared with NC group, *P<0.05; **P<0.01.
Figure 3.10 The influence of AR/ARL inhibitor on HCT116 cells

The survival rate of HCT116 cells deceased to around 90% when the concentration of fidarestat and tolrestat reached 100μmol/L. Fidarestat and tolrestat had no effect on the survival rate of HCT116 cells when the concentration was no higher than 50μmol/L. Compared with NC group, *P<0.05.
Figure 3. The influence of AR/ARL inhibitor on CaCo2 cells

The survival rate of CaCo2 cells was over 90% even when the concentration of fidarestat and tolrestat reached 100\(\mu\)mol/L. Fidarestat and tolrestat had no effect on the survival rate of CaCo2 cells when the concentration was no higher than 100\(\mu\)mol/L.
3.3.4 The effects of AR/ARL inducers on HT29 cells and SW40 cells

MTT experiment was done to determine the survival rate of HT29 and SW40 cancer cells in the presence of different concentrations of AR/ARL inducers, MG-132 and bortezomib. The concentrations of MG-132 used were 40, 20, 10, 5 and 2.5 μmol/L and the concentrations of bortezomib used were 0.4, 0.2, 0.1, 0.05 and 0.025 μmol/L.

The results of the experiments show that, when the concentration of MG-132 was 40μmol/L, the survival rate of SW480 cells decreased to about 70%, however, the survival rate of HT29 cells was still more than 90%. It seemed that SW480 was more sensitive to MG-132 than HT29 cells. MG-132 did not affect the survival rate of HT29 and SW480 cancer cells when the concentration was less than 20μmol/L. (Figure 3. 12)

On the other hand, bortezomib dramatically decreased the survival rate of SW480 cells to about 70%, but the survival rate of HT29 cells remained at over 90% when the concentration was 0.4μmol/L. Bortezomib did not affect the survival rate of HT29 and SW480 cancer cells when the concentration was less than 0.2μmol/L. (Figure 3.13)

As a result, the concentrations of MG-132 and bortezomib used in the following experiments were in the range of 20, 10, 5μmol/L and 0.2, 0.1, 0.05μmol/L.
Figure 3.12 The influence of MG-132 on HT29 and SW480 cells

When the concentration of MG-132 was 40μmol/L, the survival rate of SW480 cells decreased to about 70%, however, the survival rate of HT29 cells was more than 90%. MG-132 did not affect the survival rate of HT29 and SW480 cancer cells when the concentration was less than 20μmol/L. Compared with NC group, *P<0.05; **P<0.01.
Figure 3.13 The influence of Bortezomib on HT29 and SW480 cells

Bortezomib reduced the survival rate to about 70% of SW480 cells, but remained at over 90% of HT29 cells when the concentration was 0.4μmol/L. Bortezomib did not affect the survival rate of HT29 and SW480 cancer cells when the concentration was less than 0.2μmol/L. Compared with NC group, *P<0.05; **P<0.01.
3.3.5 The effects of 2DG cytotoxicity on 2DG sensitive cells in the presence of AR/ARL inhibitors

It had been reported that fidarestat only inhibit AR activity, while tolrestat could inhibit both AR and ARL activities (Endo, et al., 2010). The survival rates of 2DG-sensitive cancer cells were tested by MTT experiment after added 2DG together with fidarestat or tolrestat.

The results show that compared with the 2DG group (Only added 2DG), the survival rate was significantly increased to about 60%, 55% and 55% respectively after added 40, 20, 10 μmol/L of fidarestat to 2DG treated HepG2 cells. Similarly, the survival rate was significantly increased to about 70%, 60% and 55% respectively after added 40, 20, 10 μmol/L of tolrestat to 2DG treated HepG2 cells. (Figure 3.14)

Similarly, the survival rate was significantly increased to about 70%, 60% and 60% respectively after added 40, 20, 10 μmol/L of fidarestat to 2DG treated SKOV3 cells, and the survival rate was significantly increased to about 80%, 75% and 70% respectively after added 40, 20, 10 μmol/L tolrestat to 2DG treated SKOV3 cells. (Figure 3.15)

Tolrestat was more efficient than fidarestat in protecting HepG2 and SKOV3 cells against 2DG toxicity. This was not a non-specific effect of the inhibitors because fidarestat, which only inhibit AR protein, was ineffective in protecting cancer cells that overexpress both AR and ARL. In general, the AR/ARL inhibitor could significantly protected HepG2 and SKOV3 cells against 2DG toxicity. The protection by the AR/ARL inhibitors was a dose-dependent.
Figure 3.14 Survival rates of HepG2 cells when added 2DG together with AR/ARL inhibitor

Compared with 2DG group, the survival rate was significantly increased to about 60%, 55% and 55% respectively after added different concentrations of fidaresstat to 2DG induced HepG2 cells; the survival rate was significantly increased to about 70%, 60% and 55% respectively after added different concentrations of tolrestat to 2DG induced HepG2 cells. The AR/ARL inhibitor could significantly protected HepG2 cells against the 2DG toxicity. Compared with 2DG group, *P<0.05; **P<0.01.
Figure 3. 15 Survival rates of SKOV3 cells when added 2DG together with AR/ARL inhibitor

Compared with 2DG group, the survival rate was significantly increased to about 70%, 60% and 60% respectively after added different concentrations of fidarestat to 2DG induced SKOV3 cells; the survival rate was significantly increased to about 80%, 75% and 70% respectively after added tolrestat to 2DG induced SKOV3 cells. The AR/ARL inhibitor could significantly protected SKOV3 cells against the 2DG toxicity. Compared with 2DG group, **P<0.01.
On the other hand, when fidarestat and tolrestat (40, 20 and 10 μmol/L) were added to 2DG treated HCT116 and CaCo2 cells which only over-express AR, the cellular sensitivity to 2DG was significantly reduced. Compared with 2DG control group (Only added 2DG), the survival rate was significantly increased after added different concentrations of fidarestat or tolrestat to 2DG induced HCT116 cancer cells. (Figure 3.16)

In CaCo2 cells, compared with 2DG control group (Only added 2DG), the survival rate was significantly increased to after added different concentrations of fidarestat or tolrestat to 2DG induced CaCo2 cancer cells. (Figure 3.17)

Tolrestat was almost same efficient as fidarestat in protecting HCT116 and CaCo2 cells against 2DG toxicity. The reason was that both HCT116 and CaCo2 cells only over-expressed AR protein. The AR/ARL inhibitor can significantly protected HCT116 and CaCo2 cells against the 2DG toxicity. This phenomenon was a dose-dependent.
Figure 3. Survival rates of HCT116 cells when added 2DG together with AR/ARL inhibitor.

Compared with 2DG control group (Only added 2DG), the survival rate was significantly increased after added different concentrations of fidarestat or tolrestat to 2DG induced HCT116 cancer cells. The AR/ARL inhibitor can significantly protected HCT116 cells against the 2DG toxicity. Compared with 2DG group, **P<0.01.
Figure 3.17 Survival rates of CaCo2 cells when added 2DG together with AR/ARL inhibitor

Compared with 2DG control group (Only added 2DG), the survival rate was significantly increased after added different concentrations of fidarestat or tolrestat to 2DG induced CaCo2 cancer cells. The AR/ARL inhibitor can significantly protected CaCo2 cells against the 2DG toxicity. Compared with 2DG group, **P<0.01.
3.3.6 Depletion of GSH level in 2DG treated cells was restored by AR/ARL inhibitors

The effect of AR/ARL inhibition on the levels of GSH in 2DG treated cells was determined. As shown in Figure 3.18, in HepG2 and SKOV3 cancer cells, the GSH level in the NC group was about 30 nmol/mg proteins, and the level dramatically decreased to about 12 nmol/mg proteins after 2DG treatment for 48h. However, the GSH levels of in the 2DG treated HepG2 and SKOV3 cells were significantly restored to about 18 nmol/mg protein when fidarestat was added. Similarly, the GSH levels were restored to about 25 nmol/mg protein in the presence of tolrestat. Compared with 2DG group, tolrestat significantly restored the GSH level reduced by 2DG in HepG2 and SKOV3 cells; however fidarestat restored the GSH level less compared with tolrestat in HepG2 and SKOV3 cells. This is because HepG2 and SKOV3 cells over-expressed both AR and ARL proteins, but fidarestat can only inhibit AR activity.

In a similar way, fidarestat and tolrestat significantly restored the drop of GSH level induced by 2DG in HCT116 and CaCo2 cells compared with 2DG group. The GSH level was increased from about 15 to around 25 nmol/mg proteins respectively after added fidarestat and tolrestat. Fidarestat was almost same efficient as tolrestat in restoring the levels of GSH in HCT116 and CaCo2 cells which only over-express AR protein. (Figure 3.19)

In conclusion, AR/ARL inhibitors can restore the drop of GSH level induced by 2DG in 2DG-sensitive cancer cells, indicating that the decrease in GSH level is due to the activities of AR/ARL.
Figure 3. 18 GSH level of HepG2 and SKOV3 cells when added 2DG together with AR/ARL inhibitor

Compared with 2DG group, tolrestat significantly restored the levels of GSH in 2DG treated HepG2 and SKOV3 cells. However, fidarestat restored the GSH level more slightly less compared with tolrestat. Compared with NC group, ##$P<0.01$; compared with 2DG group, **$P<0.01$. 
Figure 3. 19 GSH level of HCT116 and CaCo2 cells when added 2DG together with AR/ARL inhibitor

Compared with 2DG group, the GSH level was significantly increased after added fidarestat and tolrestat. Fidarestat was as almost same efficient as tolrestat in restoring the drop of GSH in HCT116 and CaCo2 cells. Compared with NC group, **P<0.01; compared with 2DG group, *P<0.01.
3.3.7 The effect of increasing AR/ARL levels on 2DG toxicity in 2DG non-sensitive cells

HT 29 and SW480 are 2DG non-sensitive cells. We want to determine, by MTT assays, their sensitivity to 2DG when their AR/ARL levels are increased by MG-132 and bortezomib.

The results show that compared to 2DG group (Only added 2DG), the survival rate was significantly decreased to about 55%, 70% and 80% respectively in HT29 cells when 20, 10 and 5 μmol/L of MG132 was added to the media. In SW480 cells, the survival rate was decreased to about 60%, 70% and 85% respectively when 20, 10 and 5 μmol/L of MG-132 was added. (Figure 3.20)

Similarly, in HT29 cells, compared with 2DG group (Only added 2DG), the survival rate was significantly decreased to about 65%, 75% and 85% respectively when 0.2, 0.1 and 0.05 μmol/L of bortezomib was added to the 2DG-treated cells. In SW480 cells, the survival rate was decreased to about 75%, 80% and 85% respectively when 0.2, 0.1 and 0.05 μmol/L of bortezomib was added. (Figure 3.21)

These results indicated that AR/ARL inducer could significantly make the 2DG non-sensitive cells more sensitive to 2DG. This phenomenon was dose-dependent.
Figure 3. 20 Survival rates of HT29 cells and SW480 cells when added 2DG together with MG-132

Compared with 2DG group, the survival rate was significantly dropped in HT29 cells and SW480 cells after added 2DG together with different concentrations of MG-132. Compared with 2DG group, **P<0.01.
Figure 3. 21 Survival rates of HT29 cells and SW480 cells when added 2DG together with bortezomib

Compared with 2DG group, the survival rate was significantly dropped in HT29 cells and SW480 cells after added 2DG together with different concentrations of bortezomib. Compared with 2DG group, **P<0.01.
3.3.8 The expression levels of AR/ARL protein in HT29 and SW480 cells in the presence of AR/ARL inducers

The levels of AR/ARL protein in HT29 and SW480 cells induced by MG-132 and bortezomib were determined by western blot experiments. The concentrations of MG-132 used were 0, 1.15, 2.5, 5, 10 and 20 μmol/L; the concentrations of bortezomib used were 0, 0.0125, 0.025, 0.05, 0.1 and 0.2 μmol/L.

After using MG-132 to induce HT29 cells and SW480 cells, the expression of both AR and ARL proteins was significantly increased. In HT29 cells, MG-132 increased more ARL protein than AR protein. The expression of ARL protein increased about 8 times and the expression of AR protein increased about 4 times after induction by 20μmol MG-132. (Figure 3.22)

The expression of AR and ARL proteins in SW480 cells induced by MG-132 increased less compared to that of the HT29 cells. In SW480 cells, MG-132 increased more AR protein than ARL protein. The expression of AR protein increased by about 12 times and the expression of ARL protein increased by about 5 times after induction by 20μmol of MG-132. (Figure 3.23)

These results indicate that MG-132 can significantly increase the expression of AR/ARL protein in HT29 and SW480 cancer cells.
In HT29 cells, MG-132 increased more ARL protein than AR protein. The expression of ARL protein increased about 8 times and the expression of AR protein increased about 4 times after induction by 20μmol of MG-132. Compared with 0 group, **p<0.01. (A) AR/ARL band of molecular weight ~ 36kDa and the β-actin band of molecular weight ~ 42kDa. (B) The histograms showing the optical density of the bands after measurement by densitometer and analyzed using the software ImageJ.
Figure 3. The expression of AR and ARL in SW480 cells induced by MG-132

In SW480 cells, MG-132 increased more AR protein than ARL protein. The expression of AR protein increased about 12 times and the expression of ARL protein increased about 5 times after induced by 20μmol MG-132. Compared with 0 group, **p<0.01. (A) AR/ARL band of molecular weight ~ 36kDa and the β-actin band of molecular weight ~ 42kDa. (B) The histograms showing the optical density of the bands after measurement by densitometer and analyzed using the software ImageJ.
Similarly, after using another kind of AR/ARL inducer, bortezomib, to induce HT29 cells and SW480 cells, the levels of AR and ARL proteins were significantly increased. In HT29 cells, bortezomib significantly increased both AR and ARL proteins. The expression of AR protein increased by about 4 times and the expression of ARL protein increased by about 6 times after induced by 0.2μmol bortezomib. (Figure 3.24)

However, in SW480 cells, bortezomib had very limited effect on the expression of AR and ARL proteins compared with that in HT29 cells. In SW480 cells, bortezomib only increased the expression of AR protein about 7 times after induction by 0.2μmol bortezomib, but did not significantly increase the expression of ARL protein. (Figure 3.25)

These results suggested that increasing the level of AR/ARL in 2DG non-sensitive cancer cells made these cells become more sensitive to 2DG.
Figure 3. 24 The expression of AR and ARL in HT29 cells induced by bortezomib

In HT29 cells, bortezomib increased the expression of AR protein by about 4 times and the expression of ARL protein increased by about 6 times after induction by 0.2 μmol bortezomib. Compared with 0 group, **P<0.01. (A) AR/ARL band of molecular weight ~36kDa and the β-actin band of molecular weight ~42kDa. (B) The histograms showing the optical density of the bands after measurement by densitometer and analyzed using the software ImageJ.
Figure 3. 25 The expression of AR and ARL in SW480 cells induced by bortezomib

In SW480 cells, bortezomib increased the expression of AR protein about 7 times after induction by 0.2μmol bortezomib, but did not increase the expression level of ARL protein. Compared with 0 group, **P<0.01. (A) AR/ARL band of molecular weight ~ 36kDa and the β-actin band of molecular weight ~ 42kDa. (B) The histograms show the optical density of the bands after measurement by densitometer and analyzed using the software ImageJ.
3.4 Discussion

Since Warburg showed cancer cells had increased glycolysis with the concomitant increased production of lactate even in aerobic conditions, some researchers attempted to inhibit cancer growth by inhibiting glycolysis. 2DG is thought to be able to induce a state of glucose deprivation by competing with glucose transporter for uptake and the metabolism (Aft, et al., 2002). Several studies indicated that the relationship between glucose deprivation and cytotoxicity might rely by the concentration of hexokinase (Danial, et al., 2003), which constitutes a target of 2DG (Cay, et al., 1992). It was also suggested that glucose kinase interacts both with glycolysis and apoptosis in mouse liver mitochondria (Danial, et al., 2003). On the other hand, there were some reports indicated that 3-bromopyruvate induced hexokinase inhibition could cause cytotoxicity in human leukemia and lymphoma cells lines (Xu, et al., 2005). Hexokinase inhibition caused the translocation of pro-apoptotic factor and then activating the release of cytochrome C and the caspase cascade (Halicka, Ardelt, Li, Melamed, & Darzynkiewicz, 1995). However, based on the facts that 2DG only inhibit glycolysis by 15-40% (Kurtoglu, Gao, & Shang, et al., 2007) and the fact that even though 2FDG is a better inhibitor of glycolysis, it is less effective in killing cancer cells (Kelloff, et al., 2005), we proposed that cytotoxicity effect of 2DG in cancer cells was due to oxidative stress by AR/ARL activity. This thesis was to verify the novel hypothesis.

The six cell lines used in our experiments were widely used in cancer research to study cancer mechanism and for the development of anti-cancer drugs (Desjardins, et al., 2014; Magdeldin et al., 2014; Zhu et al., 2014; Wynn, Consul, Merajver, & Schnell,
HepG2 cells were spindle shaped, irregular and in disorderly arrangement. HepG2 and SKOV3 cells grow very quickly and cells adherent was unstable so that digestion time was 1~2 minutes. Sub-culture ratio was 1:4 to 1:6 (Busch, Barnhart, Martin, Flanagan, & Jackson, 1990; Schardt, et al., 1993; Morimoto, Yonehara, & Bonavida, 1993; X. Zhang, Minale, Zampella, & Smith, 1997); HCT116 and CaCo2 cells were spindle, irregular and disorder arrangement. Sub-cultivation ratio was 1:3 to 1:5 (Geetha, Nair, Latha, & Remani, 2012; Foley, Pantano, Ciolino, & Mawe, 2007); SW480 cells were nearly round and in disorderly arrangement and HT29 cells grew with no particular shape and were prone to aggregate. These two cancer cells grew slowly and cells adherent was stable. The digestion time of SW480 and HT29 cells was about 5~10 minutes and sub-cultivation ratio was 1:2 to 1:4 (Adachi, et al., 1987; Augustine, et al., 2014; Didier, et al., 1996; Jolla, Bützow, Fukushima, Twardzik, & Ruoslahti, 1993). The logarithmic growth phase cells were used for the experiments. The best cells density for freezing cells was $1\sim5 \times 10^6$ cells/ml. Cells with good growth state ensured the accurate and reliable of experimental results.

3.4.1 The sensitivity to 2DG in cancer cells correlated to their levels of AR/ARL protein

In our research, four kinds of 2DG sensitive cancer cells (HepG2 cells, HCT116 cells, CaCo2 cells and SKOV3 cells) and two kinds of 2DG non-sensitive cancer cells (HT29 cells and SW480 cells) were found according to the cells survival rate as determined by MTT experiments. As the growth rate of different cells was varies,
HepG2, HCT116, CaCo2 or SKOV3 cells were inoculated as 10000 cells per wells, and HT29 and SW480 cells were inoculated as 12000 cells per wells. In this experiment, the highest concentration of 2DG induced cancer cells was 40mmol/L, the concentration was determined by pre-experiment and the result of other researches (Aykin-Burns, Ahmad, Zhu, Oberley, & Spitz, 2010; Shutt, et al., 2010).

In the western blot experiment, the expression levels of both AR and ARL proteins were high in HepG2 cells and SKOV3 cells which were 2DG sensitive; HCT116 and CaCo2 cells, which were also 2DG sensitive, only over-expressed AR protein;. On the other hand, the levels of AR/ARL proteins were very low in HT29 and SW480 cells which were 2DG non-sensitive. Therefore, there is a direct correlation between the cells’ sensitivity to 2DG and the expression level of AR/ARL proteins.

The reason for the over-expression of AR/ARL in some cancers cells was not very clear. AR is effective in reducing toxic aldehydes including methylglyoxal (Simons, Mattson, Dornfeld, & Spitz, 2009) and 4-hydroxynonenal (Lin, et al., 2003). Since cancer cells with higher metabolic rate might produce more of these toxic metabolites, over-expression of AR might promote cell survival by neutralizing these aldehydes (Bailey, 1998). ARL has similar substrate specificity as AR. Increased level of ARL in cancers is also thought to detoxify carbonyls (Perumal, Solomon, & Jayanth, 2009).

3.4.2 Inhibition AR/ARL activity protects cancer cells against 2DG cytotoxicity

The AR/ARL was widely recognized to be involved in the pathogenesis of diabetic complications. In our hypothesis, the AR/ARL played an important role on
cytotoxicity of 2DG in cancer cells. In order to verify the hypothesis, the cytotoxicity of 2DG on HepG2, SKOV3, HCT116 and CaCo2 cells was investigated when the activities of AR/ARL protein were inhibited.

Two kinds of inhibitors (fidarestat and tolrestat) were used in this research. AR inhibitors have been considered as potential therapeutic drugs for diabetic complications. Most AR inhibitors can be divided into several groups: carboxylic acid (Tolrestat and Fidarestat), cyclic imides (Minalrestat) and flavonoids (Myricetin) (Stefek, et al., 2008). Among the carboxylic acids, tolrestat was considered as most potent inhibitor for AR protein (Endo, et al., 2010). According to crystallographic and kinetic studies, it was reported that tolrestat can bind to the active site of AR and competitively inhibits the NADP-linked geraniol oxidation to the AR’s substrate (Endo, et al., 2010; Gallego, et al., 2007). Fidarestat was used in clinical trial to treat diabetic complications, and has been implicated as a pivotal player in inflammatory pathologies (Pandey, 2015). Fidarestat inhibits only AR, while tolrestat can inhibit both AR and ARL (Endo, et al., 2010). The low selectivity of most ARIs might be the result of the similarity of tertiary structures of AR and ARL proteins including active sites (Hamada, & Nakamura, 2004; Zhang, et al., 2013). (Figure 3.26)

In this experiment, the cells was pretreated with fidarestat or tolrestat for 24h, and then added 2DG and incubated at 37°C, 5%CO₂ for additional 48 hours. It was reported that the pretreated time can be 4~10 hours, the pretreated time used in our experiment was 24 hours according to pre-experiment results (Shoeb, Yadav, Srivastava, & Ramana, 2011; Yadav, Srivastava, & Ramana, 2012). In our research, tolrestat was
more efficient than fidarestat in protecting HepG2 and SKOV3 cells against 2DG toxicity. This was because fidarestat, which only inhibit AR protein, was ineffective in protecting cells that overexpress both AR and ARL. However, tolrestat was just as efficient as fidarestat in protecting HCT116 and CaCo2 cells which only over-express AR protein.

On the other hand, AR/ARL inhibitor significantly restored the decrease of GSH induced by 2DG in 2DG sensitive cancer cells. These results indicate that the depletion of GSH in 2DG treated cells is due to AR/ARL activities.

3.4.3 Increasing the cellular level of AR/ARL made 2DG non-sensitive cancer cells became sensitive to 2DG

HT29 and SW480 expressed low levels of AR/ARL, and they are not sensitive to 2DG. We want to find out if these cells would become sensitive to 2DG when the levels of AR/ARL are increased. MG-132 (carbobenxoxy-Leu-Leu-leucinal) and bortezomib were used as AR/ARL inducers. They are kind of proteasome inhibitors. MG-132 is a peptide aldehyde which is able to inhibit different types of proteases. MG-132 had been shown to inhibit multiple peptidases (Tsubuki, Saito, Tomioka, Ito, & Kawashima, 1996). Bortezomib (Velcade), a dipeptide boronic acid derivative, can induce cytotoxic effects in some cancer cells (Russo, et al., 2001; Lenz, 2003). It had been shown that both MG-132 and bortezomib could increase the level of AR and ARL protein and mRNA in HT29 and SW480 cancer cells. Proteasome inhibitors had been reported to increase the expression of nuclear factor-erythroid 2 related factor 2
(Nrf2)-regulated genes. Several members of the AKR superfamily are regulated via Nrf2-dependent pathways. The level of AR/ARL could be increased with Nrf2-regulated genes ungraduated (Ebert, Kisiela, Wsol, & Maser, 2011). (Figure 3.27)

The results of our experiments show that the survival rate of HT29 and SW480 cells decreased significantly by the treatment of 2DG when these cells were pretreated with different concentrations of MG-132 or bortezomib. These results indicate that AR/ARL inducer, which increased the expression of AR/ARL in cancer cells, made the 2DG non-sensitive cells become sensitive to 2DG.

3.5 Conclusion

We proposed that the cytotoxic effect of 2DG in cancer cells was due to AR and/or ARL activity, not glucose deprivation. In this chapter, results of the experiments confirmed that the cytotoxic effect of 2DG in cancer cells is due to depletion of GSH induced by the reduction of 2DG by AR and/or ARL which are often overexpressed in cancer cells. This was supported by several lines of evidences: (1) levels of AR/ARL in cancer cells were correlated to their sensitivity to 2DG; (2) inhibition of AR/ARL protected 2DG sensitive cancer cells against 2DG cytotoxicity; (3) AR/ARL inhibitor restored the decrease of GSH level induced by 2DG; (4) increasing AR/ARL made 2DG non-sensitive cancer cells more sensitive to 2DG. Our findings better explain the cytotoxicity effect of 2DG in cancer cells than the inhibition of glycolysis model.
Tolrestat and fidarestat could bind to the active site of AR/ARL so that they competitively inhibit the NADP-linked geraniol oxidation to the AR/ARL substrate. Fidarestat only inhibited AR, while tolrestat can inhibit both AR and ARL proteins.
MG-132 and bortezomib which were used as AR/ARL inducer are proteasome inhibitors. Proteasome inhibitors could increase the expression of AR/ARL protein via up-regulated Nrf2-regulated genes.
Chapter 4 Better substrates for AR/ARL than 2DG are better anti-cancer agents than 2DG

4.1 Introduction

The previous results confirmed that 2DG killed cancer cells due to the activity of AR/ARL enzymes in cancer cells. However, 2DG is not an efficient substrate for AR/ARL and does not kill cancer cells efficiently. If our hypothesis is correct, better substrates for AR/ARL enzymes should have better anti-cancer effect than 2DG. Better substrates for AR/ARL, diacetyl and glyceraldehyde, were used to further confirm our hypothesis in this chapter.

The cytotoxic effect of glyceraldehyde and diacetyl was tested by MTT experiments. In order to further develop better AR/ARL substrates as anti-cancer drugs, animal models were used to verify the anti-cancer effect of glyceraldehyde and diacetyl. ICR mice were first used to determine LD50 of glyceraldehyde and diacetyl, and then human cancer xenograft nude mice models were used to evaluate their anti-tumor effects in vivo.

4.2 Objective

The objective of this chapter is to study the anti-cancer effect of glyceraldehyde and diacetyl, which are better substrates of AR/ARL than 2DG. These will lead to develop more efficient drugs for cancer treatment.
4.3 Results

4.3.1 The cellular sensitivity to glyceraldehyde in six kinds of cancer cells.

The survival rates of HepG2, SKOV3, HCT116, CaCo2, HT29 and SW480 cells in the presence of different concentrations of glyceraldehyde (5, 2.5, 1.25, 0.625 and 0.313 mmol/L) for 24 and 48 hours were determined by MTT assays.

The results show that in HepG2, SKOV3, HCT116 and CaCo2 cells, the survival rate dramatically decreased when glyceraldehyde was added to the medium for 24 hours and 48 hours. After adding 5mmol/L glyceraldehyde for 48 hours, the survival rate of these four kinds of cancer cells were dramatically decreased to about 3%. HepG2, SKOV3, HCT116 and CaCo2 cells were considered as glyceraldehyde sensitive cancer cells.

However, the survival rate of SW480 cells and HT29 cells in the presence of glyceraldehyde decreased much less than the than other cancer cells. After adding 5mmol/L glyceraldehyde for 48h, the survival rate of SW480 and HT29 cells were decreased to about 50%, which was much higher than the glyceraldehyde sensitive cancer cells. Therefore, SW480 cells and HT29 cells were considered as glyceraldehyde non-sensitive cancer cells. (Figure 4.1, Figure 4.2)

These results indicate that, similar to 2DG, glyceraldehyde also preferentially kills cells that have high levels of AR/ARL. Glyceraldehyde is more efficient than 2DG in killing cancer cells that have high levels of AR/ARL, and that cells which over-express AR and/or ARL were more sensitive to glyceraldehyde than cells with low levels of AR and ARL.
**Figure 4.1 The toxicity of glyceraldehyde on six cancer cells in 24 hours**

The survival rates of HT29 and SW480 cells were much higher than HepG2, SKOV3, HCT116 and CaCo2 cells after added glyceraldehyde for 24 hours. HepG2, SKOV3, HCT116 and CaCo2 cells were considered as glyceraldehyde sensitive cancer cells; SW480 and HT29 cells were considered as glyceraldehyde non-sensitive cancer cells. Compared with NC group, **P<0.01.**
The survival rates of HT29 and SW480 cells were much higher than HepG2, SKOV3, HCT116 and CaCo2 cells after added glyceraldehyde for 48 hours. HepG2, SKOV3, HCT116 and CaCo2 cells were considered as glyceraldehyde sensitive cancer cells; SW480 and HT29 cells were considered as glyceraldehyde non-sensitive cancer cells. Compared with NC group, *P<0.05; **P<0.01.
4.3.2 The cellular sensitivity to diacetyl in six kinds of cancer cells.

The survival rates of HepG2, SKOV3, HCT116, CaCo2, HT29 and SW480 cells in the presence of different concentrations of diacetyl (10, 5, 2.5, 1.25 and 0.625mmol/L) for 24 and 48 hours were determined by MTT experiments.

Similar to the results of the glyceraldehyde experiments, the survival rates of HepG2, SKOV3, HCT116 and CaCo2 cells also dramatically decreased when diacetyl added to the medium. After added 10mmol/L of diacetyl for 48 hours, the survival rate of these four kinds of cancer cells dramatically decreased to about 5%. These four kinds of cancer cells were therefore considered as diacetyl sensitive cancer cells.

On the other hand, the survival rate of SW480 cells and HT29 cells decreased much less than the four diacetyl sensitive cells. After added 10mmol/L of diacetyl for 48 hours, the survival rates of these cancer cells only decreased to about 25-40%. As a result SW480 cells and HT29 cells were considered diacetyl non-sensitive cancer cells. (Figure 4.3, Figure 4.4)

These results indicate that diacetyl is more efficient than 2DG in killing cancer cells that have high levels of AR/ARL, and that cells which over-express AR and/or ARL were more sensitive to diacetyl than cells with low levels of AR and ARL.
Figure 4.3 The toxicity of diacetyl on six cancer cells in 24 hours

In the presence of different concentrations of diacetyl for 24 hours, the survival rates of HT29 and SW480 cells were higher than HepG2, SKOV3, HCT116 and CaCo2. HepG2, SKOV3, HCT116 and CaCo2 cells were considered as diacetyl sensitive cancer cells; and SW480 and HT29 cells were considered as diacetyl non-sensitive cancer cells. Compared with NC group, *P<0.05; **P<0.01.
Figure 4.4 The toxicity of diacetyl on six cancer cells in 48 hours

In the presence of different concentrations of diacetyl for 48 hours, the survival rates of HT29 and SW480 cells were much higher than HepG2, SKOV3, HCT116 and CaCo2 cells. HepG2, SKOV3, HCT116 and CaCo2 cells were considered as diacetyl sensitive cancer cells; and SW480 and HT29 cells were considered as diacetyl non-sensitive cancer cells. Compared with NC group, **P<0.01.
4.3.3 Enzyme activity assays of glyceraldehyde, diacetyl and 2DG.

In order to test our hypothesis that better AR/ARL substrate can kill cancer cells more efficiently, the enzyme activity assay of AR/ARL substrates (glyceraldehyde, diacetyl and 2DG) was carried out. Enzyme activities were determined by monitoring the rate of oxidation of the co-factor NADPH. The faster NADPH is oxidized, the higher the catalytic efficiency of AR/ARL.

The results of the enzyme activity assays show that, glyceraldehyde was the most efficient substrate for AR, which oxidized about 1700 nmol NADPH per min per mg protein, followed by diacetyl (about 700 nmol) and 2DG (about 150 nmol). Diacetyl was the most efficient substrate for ARL, which oxidized about 2300 nmol NADPH per min per mg protein, followed by glyceraldehyde (about 1800 nmol) and 2DG (about 100 nmol).

In conclusion, glyceraldehyde was the most efficient substrate for AR; diacetyl was the most efficient substrate for ARL protein and 2DG was the least efficient substrate for both AR and ARL proteins. (Figure 4.5)
Figure 4.5 Enzyme activity assay of glyceraldehyde, diacetyl and 2DG

Glyceraldehyde was the most efficient substrate for AR; diacetyl was the most efficient substrate for ARL; 2DG was the least efficient substrate for both AR and ARL.
4.3.4 Cytotoxicity of AR/ARL substrates depended on their catalytic efficiency for AR/ARL.

The cytotoxic effect of glyceraldehyde, diacetyl and 2DG on cancer cells for 24 and 48 hours was determined.

As shown in Figure 4.6, the cytotoxicity of glyceraldehyde on HepG2, SKOV3, HCT116 and CaCo2 was greater than diacetyl, followed by 2DG after treatment with 48 hours. The cytotoxicity results after treatment with 24 hours was as similar as 48 hours. (Figure 4.7)

In HCT116 and CaCo2 cancer cells, which only over-express AR protein, the cytotoxicity effect of glyceraldehyde, diacetyl and 2DG was consistent with their catalytic efficiency for AR. On the other hand, HepG2 and SKOV3 cancer cells over-expressed both AR and ARL enzymes. Although diacetyl was most efficient substrate for ARL, diacetyl had much lower catalytic efficiency for AR than glyceraldehyde. Glyceraldehyde was the most efficient substrate for AR and good substrate for ARL at the same time. Over all, glyceraldehyde was more efficient in killing HepG2 and SKOV3 cancer cells than diacetyl, and 2DG was least efficient in killing these cancer cells.

In conclusion, the cytotoxicity of AR/ARL substrates was consistent with their catalytic efficiency for AR/ARL. The more efficient they served as the substrates for AR/ARL, the more efficient they kill cancer cells that over-expressed these enzymes.
The cytotoxicity of glyceraldehyde on cancer cells was greater than diacetyl, followed by 2DG after treatment with 48 hours. The severity of cytotoxicity of the AKR substrates correlates to their catalytic efficiency for AR/ARL. Compared with same concentration of 2DG group, **P<0.01. (A) The concentration of glyceraldehyde, diacetyl and 2DG used was 5.00mmol/L. (B) The concentration of glyceraldehyde, diacetyl and 2DG used was 2.50mmol/L.
The cytotoxicity of Glyceraldehyde on cancer cells was greater than diacetyl, followed by 2DG after treatment with 24 hours. The severity of cytotoxicity of the AKR substrates correlates to their catalytic efficiency for AR/ARL. Compared with same concentration of 2DG group, **P<0.01. (A) The concentration of glyceraldehyde, diacetyl and 2DG used was 5.00mmol/L. (B) The concentration of glyceraldehyde, diacetyl and 2DG used was 2.50mmol/L.
4.3.5 The level of GSH on cancer cells treated by glyceraldehyde/diacetyl

As we proposed that cytotoxicity of AR/ARL substrates was due to the depletion of GSH, the GSH level on cancer cells treated with glyceraldehyde (5mmol/L) or diacetyl (5mmol/L) for 48 hours were determined.

The results show that both glyceraldehyde and diacetyl can significantly reduce the levels of GSH in HepG2, SKOV3, HCT116 and CaCo2 cancer cells which over-express AR and/or ARL protein. In HepG2 cells, GSH level was significantly reduced to about 10 nmol/mg protein after glyceraldehyde treatment and to about 9 nmol/mg protein after diacetyl treatment. In SKOV3 cells, GSH level was significantly reduced to about 10 and 13 nmol/mg protein respectively, after treatment with glyceraldehyde or diacetyl. In HCT116 cells, GSH level was significantly reduced to about 10 and 13 nmol/mg protein respectively, after treatment with glyceraldehyde or diacetyl. In CaCo2 cells, GSH level was significantly reduced to about 11 and 16 nmol/mg protein respectively, after treatment with glyceraldehyde or diacetyl.

However, in HT29 and SW480 cells, glyceraldehyde and diacetyl reduced the levels of GSH less than cancer cells that over-expressed AR/ARL. After treated with glyceraldehyde or diacetyl for 48 hours, GSH level was reduced to about 20 nmol/mg protein in both HT29 and SW480 cells. (Figure 4.8)

Therefore, the results show that Glyceraldehyde and diacetyl depleted more GSH in cancer cells which over-express AR and/or ARL than in cancer cells with low levels of AR and ARL. This is consistent with our hypothesis that the cytotoxicity of the AR/ARL substrates is due to the activities of AR/ARL in these cancer cells.
Both glyceraldehyde and diacetyl can significantly reduce the levels of GSH in HepG2, SKOV3, HCT116, and CaCo2 cancer cells, and the decrease in GSH levels was less in SW480 and HT29 cancer cells. Glyceraldehyde and diacetyl depleted more GSH in cancer cells with high levels of AR and/or ARL than in cancer cells with low levels of AR and ARL. Compared with NC group, *P<0.05, **P<0.01.
4.3.6 Inhibition of GSH synthesis enhanced the cytotoxicity of AR/ARL substrates

According to our hypothesis, GSH plays a very important role in the cytotoxicity of AR/ARL substrates. To confirm this, we determined the cytotoxicity of AR/ARL substrates when the cellular level of GSH was decreased. BSO (L-Buthionine-S,R-sulfoximine) is a GSH inhibitor of the synthesis of GSH, resulting in the depletion of GSH (Marketa K. and Petr M. et al, 2015). The survival rate of cancer cells treated with glyceraldehyde or diacetyl for 48 hours in the presence or absence of BSO was determined.

As shown in Figure 4.9, BSO (Gly+BSO group) significantly enhanced the cytotoxicity of glyceraldehyde in cancer cells. Compared with glyceraldehyde group, the survival rate of cancer cells was significantly decreased when BSO (0.3mM) was added together with glyceraldehyde (1.25mM or 0.625mM) group. (Figure 4.9)

Similarly, BSO (Dia+BSO group) significantly enhanced the cytotoxicity of diacetyl in cancer cells. Compared with diacetyl group, the survival rate of cancer cells was significantly decreased when BSO (0.3mM) was added together with diacetyl (1.25mM or 0.625mM) group. (Figure 4.10)

These results indicate that decrease of cellular GSH levels enhanced the cytotoxicity of glyceraldehyde and diacetyl in cancer cells.
The survival rate of cancer cells was significantly decreased after BSO was added for 48 hours compared with only glyceraldehyde group. BSO significantly enhanced the cytotoxicity of glyceraldehyde in cancer cells. Compared with Gly (1.25mM) group, **P<0.01; compared with Gly (0.625mM) group, ## P <0.01
Figure 4.10 BSO enhanced the cytotoxicity of diacetyl on cancer cells

The survival rate of cancer cells was significantly decreased after BSO was added for 48 hours compared with only diacetyl group. BSO significantly enhanced the cytotoxicity of diacetyl in cancer cells. Compared with Dia (1.25mM) group, **P<0.01; compared with Dia (0.625mM) group, ## P <0.01
4.3.7 Increase GSH synthesis protected cells against the cytotoxicity of AR/ARL substrates

NAC (N-Acetyl-cysteine) is a precursor of GSH synthesis, and it is commonly used to increase the cellular concentrations of GSH (Zhang, et al., 2013). The survival rate of cancer cells treated with glyceraldehyde or diacetyl for 48 hours in the presence or absence of NAC was determined.

The results show that NAC (Gly+NAC group) significantly protected the cells against the cytotoxicity of glyceraldehyde. Compared with Glyceraldehyde group, the survival rate of cancer cells was significantly increased when NAC (1mM) was added together with glyceraldehyde (2.50mM or 1.25mM) group. (Figure 4.11)

Similarly, NAC (Dia+NAC group) significantly protected the cells against the cytotoxicity of diacetyl. Compared with diacetyl group, the survival rate of cancer cells was significantly increased when NAC (1mM) was added together with diacetyl (2.50mM or 1.25mM) group. (Figure 4.12)

These results indicate that increasing the cellular levels of GSH protected the cancer cells against the cytotoxicity of glyceraldehyde and diacetyl. Together with the results in the previous section that inhibition of GSH synthesis enhanced the cytotoxicity of the AR/ARL substrates, these results show that depletion of GSH is the main mechanism of the cytotoxic effects of the AR/ARL substrates in cells that have high levels of these enzymes.
Figure 4.11 NAC protected cancer cells against the cytotoxicity of glyceraldehyde

The survival rate of cancer cells was significantly increased when NAC was added to the cells for 48 hours compared with only glyceraldehyde group. NAC significantly protected cancer cells against the cytotoxicity of glyceraldehyde. Compared with Gly (2.50mM) group, **P<0.01; compared with Gly (1.25mM) group, ## P <0.01
The survival rate of cancer cells was significantly increased when NAC was added for 48 hours compared with only diacetyl group. NAC significantly protected cancer cells against the cytotoxicity of diacetyl. Compared with Dia (2.50mM) group, **$P<0.01$; compared with Dia (1.25mM) group, ## $P<0.01$. 

**Figure 4.12** NAC protect cancer cells against the cytotoxicity of diacetyl
4.3.8 Determination of LD50 of glyceraldehyde in acute toxicity test.

In order to further develop glyceraldehyde and diacetyl as anti-cancer drugs, animal model was used to demonstrate their anti-cancer effect. LD50 of glyceraldehyde was first determined in acute toxicity test.

The dosage of intravenous administration in this experiment was 1000.0mg/kg and 2000.0mg/kg body weight. The latter was the highest concentration of glyceraldehyde solution that can be made.

No mouse died with the two concentrations of glyceraldehyde injected, indicating that LD50 of glyceraldehyde is higher than 2000mg/kg bod weight (Table 4.1). After receiving the drug through tail vein injection, some mice had the symptoms of swelling, inflammation, or discharge at the tail. The symptoms were significantly relieved after using alcohol to disinfect every day. The body weight of all mice increased normally during the study period. (Table 4.2)
### Table 4.1 Acute mortality test by glyceraldehyde in mice (n=8)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Group</th>
<th>Dose (mg/kg BW)</th>
<th>Death/Total</th>
<th>Death Rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde</td>
<td>1</td>
<td>1000.0</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2000.0</td>
<td>0/8</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 4.2 The weight change after glyceraldehyde injection in mice (\( \bar{x} \pm SD \))

<table>
<thead>
<tr>
<th>Glyceraldehyde (mg/kg)(Survival)</th>
<th>Weight on days after administration (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000(8)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>22.46±0.85</td>
</tr>
<tr>
<td>2000(8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.76±0.88</td>
</tr>
</tbody>
</table>
4.3.9 Determination of LD50 of diacetyl in acute toxicity test.

LD50 of diacetyl was determined in acute toxicity test. In this experiment, five dose groups were designed according to the results of prior experiments. When mice were given 300.0mg/kg bodyweight, no mice died. The second dose was increased to 360.0mg/kg bodyweight. At this dosage, three mice died. The dose for the third experiment was increased to 432mg/kg bodyweight and five mice died. The fourth dose was 518.4 mg/kg bodyweight and seven mice died. The dose for the fifth experiment was set to 622.1mg/kg bodyweight and all eight mice died. (Table 4.3)

Based on the above data, the LD50 for diacetyl was estimated to be about 410 mg/kg bodyweight. The surviving mice did not show any behavior changes or toxic symptoms, and bodyweight increased normally during the study period. (Table 4.4)
Table 4.3 Acute mortality test by diacetyl in mice (n=8)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Group</th>
<th>Dose (mg/kg BW)</th>
<th>Death/Total</th>
<th>Death Rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300.0</td>
<td>0/8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>360.0</td>
<td>3/8</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>Diacetyl</td>
<td>3</td>
<td>432.0</td>
<td>5/8</td>
<td>62.5</td>
</tr>
<tr>
<td>4</td>
<td>518.4</td>
<td>7/8</td>
<td>87.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>622.1</td>
<td>8/8</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4 The weight change after diacetyl injection in mice (±SD)

<table>
<thead>
<tr>
<th>Diacetyl (mg/kg)(Survival)</th>
<th>Weight on days after administration (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>300.0(8)</td>
<td>22.75±2.16</td>
</tr>
<tr>
<td>360.0(5)</td>
<td>22.84±1.75</td>
</tr>
<tr>
<td>432.0(3)</td>
<td>22.87±2.63</td>
</tr>
<tr>
<td>518.4(1)</td>
<td>20.70</td>
</tr>
</tbody>
</table>
4.3.10 Anti-cancer effect of glyceraldehyde in tumor xenografts model

To further study the anti-cancer potential of glyceraldehyde, HepG2 tumor xenograft model was developed by injecting nude mice with HepG2 cells. When the tumor size reached about 200mm$^3$, these mice were injected with 500 mg/kg glyceraldehyde for 3 weeks.

The results showed that glyceraldehyde significantly inhibited the tumor growth. Compared with the NC group, the tumor volume was significantly smaller after treatment with glyceraldehyde for 9 days (Figure 4.13). On the other hand, the body weight was very stable during the study period. The body weight of both NC group and Gly group was stable at about 22~24 g (Figure 4.14).

At the end of experiment, the mice were sacrificed and the tumors were weighted and their sizes measured. The tumor weight of Gly group was about 0.8g and the NC group was over 1.0g. Compared with NC group, tumor weight was significantly reduced after glyceraldehyde treatment. (Figure 4.15)

The results clearly indicated that glyceraldehyde had significant anti-cancer effect in HepG2 tumor xenograft model.
Figure 4.13 The tumor volume of glyceraldehyde (500mg/kg) in tumor xenografts study

The tumor volume was significantly smaller after treatment of glyceraldehyde for 9 days. Glyceraldehyde significantly inhibited the tumor growth. Compared with NC group, *P<0.05; **P<0.01.
Figure 4.14 The body weight of glyceraldehyde (500mg/kg) in tumor xenografts study

The body weight of both NC group and Gly group was stable at about 22~24g. The body weight was very stable during the study period.
Figure 4.15 The tumor weight of glyceraldehyde (500mg/kg) in tumor xenografts study

Tumor weight was significantly reduced after treatment with glyceraldehyde for 3 weeks. Glyceraldehyde had significantly anti-cancer effect in HepG2 tumor xenograft model. Compared with NC group, *P<0.05. (A) The tumor photo of glyceraldehyde in tumor xenografts study. (B) The tumor weight of glyceraldehyde in tumor xenografts study.
4.3.11 Anti-cancer effect of diacetyl in tumor xenografts model

To study the antitumor effect of diacetyl, HepG2 tumor xenografts nude mice received daily single dose injection of diacetyl (80mg/kg) via the tail vein for 3 weeks.

The results showed that diacetyl significantly inhibited the tumor growth. Compared with NC group, the tumor volume was significantly smaller after treatment with diacetyl for 9 days (Figure 4.16). On the other hand, the body weights after diacetyl treatment were similar to that of the non-treated group. The body weight of both NC group and Dia group steady increased from about 19g to around 22g (Figure 4.17).

At the end of experiment, the tumor weight of Dia group was about 0.9 g and the NC group was about 1.2 g. Compared with NC group, tumor weight was significantly reduced after diacetyl treatment at the end of test (Figure 4.18).

The result clearly indicated that diacetyl had anti-cancer effect in HepG2 tumor xenograft model, indicating that AR/ARL substrates could serve as anticancer drugs.
The tumor volume of diacetyl (80mg/kg) in tumor xenografts study

The tumor volume was significantly smaller after treatment of diacetyl for 9 days. Diacetyl significantly inhibited the tumor growth. Compared with NC group, *P<0.05; **P<0.01
Figure 4.17 The body weight of diacetyl (80mg/kg) in tumor xenografts study

The body weight of both NC group and Dia group steadily increased from about 19g to 22g. The body weight was very stable after diacetyl treatment for 3 weeks.
Figure 4.18 The tumor weight of diacetyl (80mg/kg) in tumor xenografts study

Tumor weight was significantly reduced after treatment with diacetyl for 3 weeks. Diacetyl had significantly anti-cancer effect in HepG2 tumor xenograft model. Compared with NC group, *P<0.05. (A) The tumor picture of diacetyl in tumor xenografts study. (B) The tumor weight of diacetyl in tumor xenografts study
4.4 Discussion

Our experiments confirmed that cytotoxicity of 2DG in cancer cells was due to the depletion of GSH as a consequence of the reduction of 2DG by AR/ARL. Its cancer-specific cytotoxicity is due to the fact that many cancer cells over-expressed AR/ARL. Based on this mechanism, substrates of AR/ARL that can be efficiently reduced by these two enzymes could be used to specifically kill cancer cells that over-express these enzymes. 2DG is not an efficient substrate for AR and ARL. We showed that two more efficient substrates, diacetyl and glyceraldehyde, were more effective in killing cancer cells than 2DG. This further supports our hypothesis. (Figure 4.19)

4.4.1 Cancer cells that over-express AR/ARL were more sensitive to glyceraldehyde and diacetyl than cells with low levels of AR and ARL

In our research, diacetyl and glyceraldehyde were shown to be more efficient in killing cancer cells that overexpress AR/ARL. The survival rate of cancer cells was dramatically decreased to about 3% after added 5mmol/L glyceraldehyde for 48h in HepG2, SKOV3, HCT116 and CaCo2 cells, and decreased to about 45% in SW480 and HT29 cells. Similarly, the survival rate of cancer cells were dramatically decreased to about 5% after added 10mmol/L diacetyl for 48h in HepG2, SKOV3, HCT116 and CaCo2 cells, and decreased less in SW480 and HT29 cells.
Both glyceraldehyde and diacetyl are better substrates for AR/ARL enzyme than 2DG.

Figure 4.19 Molecular structures of glyceraldehyde and diacetyl
HepG2, SKOV3, HCT116 and CaCo2 cells were considered as glyceraldehyde and diacetyl sensitive cancer cells, however SW480 and HT29 cells were considered as less-sensitive cancer cells. Glyceraldehyde and diacetyl were more efficient in killing cancer cells than 2DG. On the other hand, cancer cells which over-express AR/ARL proteins were more sensitive to glyceraldehyde and diacetyl than cancer cells with low levels of AR and ARL proteins.

4.4.2 Cytotoxicity effect of glyceraldehyde, diacetyl and 2DG correlated to their catalytic efficiency for AR/ARL

According to the enzyme assay results, 2DG was the least efficient substrate for AR and ARL. Glyceraldehyde was the most efficient substrate for AR protein, followed by diacetyl and then 2DG. In HCT116 and CaCo2 cancer cells, which only over-express AR protein, the cytotoxicity effect of these substrates was consistent with their catalytic efficiency for AR protein. Diacetyl was most efficient substrate for ARL protein, but had much lower catalytic efficiency for AR than glyceraldehyde. Glyceraldehyde was most efficient substrate for AR protein and good substrate for ARL protein at the same time. Over all, glyceraldehyde was more efficient substrate for AR/ARL than diacetyl in HepG2 and SKOV3 which over-express both AR and ARL proteins. According to the result of cytotoxicity test, glyceraldehyde was more efficient than diacetyl, and 2DG was least efficient in killing HepG2 and SKOV3 cancer cells which over-express AR and ARL proteins. These results suggested that the severity of cytotoxicity of glyceraldehyde, diacetyl and 2DG were correlated with their
catalytic efficiency for AR/ARL.

4.4.3 Cytotoxicity effect of glyceraldehyde and diacetyl was due to the depletion of GSH

The results of our experiments showed that AR/ARL activities were very critical for the cytotoxicity of their substrates. AR/ARL substrate can kill cancer cells because of depletion of GSH in the process of being reduced by AR/ARL.

GSH levels in cancer cells treated with diacetyl and glyceraldehyde were determined. Diacetyl and glyceraldehyde could significantly decrease GSH level, and the magnitude of decrease of GSH in cells that over-express AR and/or ARL enzyme was significantly higher than in cells with low levels of these enzymes. To further prove that AR/ALR substrates (diacetyl and glyceraldehyde) kill cells that overexpress AR/ARL because of the depletion of GSH, the cytotoxicity of glyceraldehyde and diacetyl in cells with decreased or increased levels of GSH was determined. First, the survival rate of cancer cells treated with diacetyl/glyceraldehyde together with BSO, an inhibitor of GSH synthesis, was determined. BSO was a drug that reversibly inhibits glutamate cysteine ligase activity, and consequently inhibited GSH synthesis, resulting in GSH level decreased (Sobhakumari, et al., 2012). The results of these experiments showed that BSO significantly enhanced the cytotoxicity of glyceraldehyde or diacetyl in cancer cells. To further confirm that decreases GSH level is important for the cytotoxicity of diacetyl/glyceraldehyde the survival rate of cancer cells treated with diacetyl/glyceraldehyde together with NAC was determined. NAC is a precursor of
GSH synthesis and commonly used to increase cellular GSH level (Tagde, Singh, Kang, & Reynolds, 2014). The results showed that NAC significantly protected cancer cells against the cytotoxicity of glyceraldehyde or diacetyl.

4.4.4 Glyceraldehyde and diacetyl, which are better substrates for AR/ARL, were effective in reducing tumor growth in human HepG2 tumor xenograft model.

Since we demonstrated that glyceraldehyde and diacetyl were more efficient than 2DG in killing cancer cells, animal experiments were done to confirm the in vitro findings.

Due to the concern that high levels of AR/ARL in the digestive tract might catalyze their substrates, the method of drug administration in animal experiment was decided to be by intravenous injections. Acute toxicity test was done first to determine a safe dosage. The results of acute toxicity test indicated that glyceraldehyde was safer than diacetyl for mice via intravenous injection. In the acute toxicity test on glyceraldehyde, there was no mortality during the observation period after administration of a single 2000mg/kg dose to mice via intravenous injection. Therefore, the LD50 of glyceraldehyde was considered to be greater than 2000mg/kg. According to the regulations of Globally Harmonized System of Classification and Labelling of Chemicals, the compound which LD50 value greater than 2000 mg/kg was considered as relatively safe on acute exposure (Sairam, & Urooj, 2014). On the other hand, in the acute toxicity test of diacetyl, mortality occurred within 30 mins when a dose of 360mg/kg or higher was administered. The LD50 of diacetyl was estimated to be
around 410 mg/kg. The surviving mice did not show any noticeable symptoms of toxicity or mortality and their bodyweights increased normally during the observation period. In acute toxicity of glyceraldehyde, some mice after drug injected had symptoms of swelling, inflammation and fever at the tail. These symptoms were significantly relieved after using alcohol to disinfect every day. It was not clear whether the inflammation was caused by the toxicity of glyceraldehyde or due to the process of injection. Inflammation at the tail did not appear in the acute toxicity tests of diacetyl.

After the LD50 of glyceraldehyde and diacetyl was determined, xenograft study was done to assess their anti-cancer effect. In the development of tumor xenograft in nude mice, tumor grew slowly in first few days after injection of HepG2 cells. It needed about 10 days for the tumor to grow to about 200 mm$^3$ and then treatment started. Mice were randomized for different experimental groups. Each treatment group had six mice and received treatment daily by tail vein injection for 3 weeks.

Based on the result of the acute toxicity tests, the dosage of the drugs was determined. However, for glyceraldehyde, no LD50 was determined because of solubility problem. The highest dose use in the toxicity test was 2000mg/kg. Therefore, 500mg/kg of glyceraldehyde, which was less than 1/4 LD50, was used in tumor xenograft study. The results showed that glyceraldehyde significantly inhibited the tumor growth and reduced the tumor weight. On the other hand, the body weight was very stable after glyceraldehyde treatment. This indicated that at the dosage used, glyceraldehyde did not affect the nude mice survival. For diacetyl, 80mg/kg diacetyl,
which was less than 1/5 LD50, was used for the tumor xenograft studies. A dose of less than 1/5 LD50 is considered as safe. The results suggested that diacetyl significantly inhibited the tumor growth and reduced tumor weight in xenograft model. Meanwhile, there was no significant difference on body weight after diacetyl treatment. These results indicate that 80mg/kg diacetyl had anti-cancer effect without significant effect on the overall health of the nude mice.

Male nude mice were used in the experiments due to concern that estrogen might influence the drug effect. Estrogen might not directly affect the drug-target interaction, but it might affect the expression of many genes, some of which might affect the drug response. According to the results of xenograft study, the tumor weight was only reduced by about 20% after 3 weeks of glyceraldehyde or diacetyl treatment. It seemed that glyceraldehyde and diacetyl was not very efficient in inhibiting tumor growth. On the other hand, previous studies showed that 2DG had no significantly anti-cancer effect on MCF-7 induced xenograft model (Tagg, et al., 2008). MCF-7 cancer cells were also considered as over-express AR/ARL protein (Heibein, Guo, Sprowl, Maclean, & Parissenti, 2012). Taken together, our tumor xenograft study results suggested that glyceraldehyde and diacetyl had significantly better anti-cancer effect than 2DG. The poor results in our in vivo studies are probably due to the fact the drug availability in vivo had not been optimized. Proper pharmacokinetic studies needed to be performed to determine the optimum delivery method and dosage. Nevertheless, our results strongly suggest that better substrates of AR/ARL could serve as effective anti-cancer drugs to treat cancers that over-express AR/ARL, and that more efficient
anti-cancer drugs could be designed based on the mechanism we proposed.

4.5 Conclusion

The results of our experiments confirmed that cytotoxicity of 2DG in cancer cells was due to AR/ARL activity. However, 2DG was not an efficient substrate for AR/ARL enzyme and did not kill cancer cells efficiently. According to our hypothesis, better substrate should have better anti-cancer effect.

The experiments in this chapter confirmed that glyceraldehyde and diacetyl, which are better substrates for AR/ARL than 2DG, were more efficient in killing cancer cells and had significant anti-cancer effect on xenograft model. This was supported by several lines of evidences: (1) glyceraldehyde and diacetyl were more toxic to cancer cells than 2DG; (2) glyceraldehyde and diacetyl reduced the level of GSH in cancer cells; (3) cytotoxicity of AKR substrates depends on the catalytic efficiency of AR/ARL; (4) inhibition of GSH synthesis enhances AKR substrates’ cytotoxicity; increase GSH synthesis protect cells against AKR substrates cytotoxicity; (5) glyceraldehyde and diacetyl had anti-cancer effect in xenografts model. These findings could be used to develop more efficient drugs for cancer treatment.
Chapter 5 Summary and future prospects

5.1 Summary

2DG is a non-metabolizable glucose analog which is currently being used in clinical trials to enhance the therapeutic effects of radiotherapy and chemotherapy of several types of cancers. It is thought to kill cancer cells by inhibiting glycolysis. However, 2DG can only inhibit glycolysis for 15-40%, which is not enough to cause glucose starvation. Further, 2FDG which is a more potent glycolysis inhibitor than 2DG is less effective than 2DG in killing cancer cells. These observations suggested that 2DG did not kill cancer cells by inhibition of glucose metabolism. In our previous study, inhibition AR activity decreased 2DG-induced oxidative stress in cardiomyocytes (Tang, et al., 2010), and AR were often over-express in many kinds of cancer cells. Based on these facts, we proposed that cytotoxicity of 2DG was due to the depletion of GSH as a consequence of reduction of 2DG by activities of AR/ARL rather than through inhibition of glycolysis.

Our research was to confirm this novel hypothesis. The results of our research clearly confirmed that the cytotoxicity of 2DG in cancer cells was due to the depletion of GSH resulting from the reduction of 2DG by AR/ARL. This better explains the cytotoxic effect of 2DG in cancer cells than the glycolysis inhibition model. Furthermore, since 2DG is not an efficient substrate for AR/ARL and does not kill cancer cell efficiently, according to our hypothesis, better substrate of AR/ARL should be more efficient in killing cancer cells. We demonstrated that Glyceraldehyde and diacetyl, which are better substrates for AR/ARL than 2DG, are indeed more effective
in killing cancer cells that have high levels of AR/ARL. Our research would lead to the development of better anti-cancer drugs.

5.2 Future prospects

In this thesis, a novel anticancer mechanism based on the activities of AR/ARL had been identified. This mechanism would lead to the development of a new class of efficient anti-cancer drugs. AR/ARL enzyme can reduce a variety of small molecular weight aldehydes. We have demonstrated that two of the AR/ARL substrates, glyceraldehyde and diacetyl, could serve as anti-cancer drugs and should be more effective than 2DG. We believe that even better anticancer drugs can be developed. It should be possible to design better substrates for AR/ARL to serve as anti-cancer drugs. Substrates preferred by ARL might be better anticancer drug candidates because unlike AR, which is expressed in almost all tissues, ARL is normally expressed only in the colon and small intestine (Busu, Atanasiu, Caldito, & Aw, 2014). This should restrict the potential undesirable side effects of the drugs. It will be better if we can find some substrates preferred by ARL than AR.

On the other hand, another group of enzymes in the family of aldo-keto reductases, the AKR1C1, AKR1C2, and AKR1C3, are also found to be over-expressed in some cancer cells, such as breast, cervical, and prostate cancers (Byrns, & Penning, 2009; Rižner, 2012). Similar to AR/ARL, these enzymes also need NADPH as co-factor. According to our hypothesis, the substrates of AKR1C1, AKR1C2 or AKR1C3 could also be used to treat cancers, which over-expressed these enzymes.
References


Liu, H., Savaraj, N., Priebe, W., & Lampidis, T. J. (2002). Hypoxia increases tumor cell sensitivity to glycolytic inhibitors: A strategy for solid tumor therapy (Model


Information in this thesis is being used for patent application. Do not reveal the content of this thesis without written permission from Shiqing Zhang and Stephen Chung.
## Appendix I

The following reagents and consumables were used in the present studies.

<table>
<thead>
<tr>
<th>Reagents/Consumables</th>
<th>Cat. No.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI Medium 1640, powder</td>
<td>31800</td>
<td>Gibco</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium, powder</td>
<td>12800</td>
<td>Gibco</td>
</tr>
<tr>
<td>McCoy’s 5A Medium</td>
<td>16600</td>
<td>Gibco</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>10270</td>
<td>Gibco</td>
</tr>
<tr>
<td>PSN</td>
<td>15640</td>
<td>Gibco</td>
</tr>
<tr>
<td>0.25% Trypsin-EDTA</td>
<td>25200</td>
<td>Gibco</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS), powder</td>
<td>21600</td>
<td>Gibco</td>
</tr>
<tr>
<td>30% Acrylamide/Bis Solution</td>
<td>1610156</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Tetramethylethlene diamine (TEMED)</td>
<td>T9281</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cytobuster protein extraction reagent</td>
<td>71009</td>
<td>Novagen</td>
</tr>
<tr>
<td>Protease inhibitor</td>
<td>539134</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>DL-buthionine-sulfoximine (BSO)</td>
<td>19176</td>
<td>Sigma</td>
</tr>
<tr>
<td>N-acetyl-cytein(NAC)</td>
<td>A7250</td>
<td>Sigma</td>
</tr>
<tr>
<td>Thiazolyl Blue Tetrazolium (MTT)</td>
<td>M2128</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>5879</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>5750</td>
<td>Sigma</td>
</tr>
<tr>
<td>Stripping buffer</td>
<td>CW0056A</td>
<td>CW Bio</td>
</tr>
<tr>
<td>75cm$^2$ flask</td>
<td>156499</td>
<td>Thermo</td>
</tr>
<tr>
<td>25cm$^2$ flask</td>
<td>156367</td>
<td>Thermo</td>
</tr>
<tr>
<td>96 well cells culture plate</td>
<td>310109008</td>
<td>LabServ</td>
</tr>
<tr>
<td>6 well cells culture plate</td>
<td>310109005</td>
<td>LabServ</td>
</tr>
<tr>
<td>50ml conical tubes</td>
<td>352070</td>
<td>FALCON</td>
</tr>
<tr>
<td>15ml conical tube</td>
<td>352096</td>
<td>FALCON</td>
</tr>
</tbody>
</table>
### Appendix II

The following solutions and buffers were used in the present studies.

1. **Prepare 10% gel for running electrophoreses**

   **Stack gel**
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli Q water</td>
<td>4.2 ml</td>
</tr>
<tr>
<td>Tris-HCl, 1M, pH 6.8</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>30% polyacrylamide</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>SDS, 10%</td>
<td>30 μl</td>
</tr>
<tr>
<td>APS, 10%</td>
<td>45 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>9 μl</td>
</tr>
</tbody>
</table>

   **Stacke gel**
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli Q water</td>
<td>4.7 ml</td>
</tr>
<tr>
<td>Tris-HCl, 1M, pH 8.8</td>
<td>6.0 ml</td>
</tr>
<tr>
<td>30% polyacrylamide</td>
<td>5.3 ml</td>
</tr>
<tr>
<td>SDS, 10%</td>
<td>80 μl</td>
</tr>
<tr>
<td>APS, 10%</td>
<td>80 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>16 μl</td>
</tr>
</tbody>
</table>

2. **10X Electrophoresis buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>30.3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>144.2 g</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
</tr>
</tbody>
</table>

   Fill up to 1L with Milli Q water; Adjusted pH to 8.3 and stored at RT

3. **1M Tris-HCl (For Lower Gel)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>15.76 g</td>
</tr>
</tbody>
</table>

   Fill up to 100ml with Milli Q water; Adjusted pH to 8.8 and stored at RT
4. **1M Tris-HCl (For Stake Gel)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>15.76g</td>
</tr>
</tbody>
</table>

Fill up to 100ml with Milli Q water; Adjusted pH to 6.8 and stored at RT

5. **1M Tris-HCl (For transfer buffer)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>15.76g</td>
</tr>
</tbody>
</table>

Fill up to 100ml with Milli Q water; Adjusted pH to 8.3 and stored at RT

6. **10% SDS**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>5g</td>
</tr>
</tbody>
</table>

Fill up to 50ml with Milli Q water; Stored at RT

7. **Transfer buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl, pH 8.3</td>
<td>50ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>200ml</td>
</tr>
<tr>
<td>Glycine</td>
<td>11.2g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>5ml</td>
</tr>
<tr>
<td>Milli Q water</td>
<td>740ml</td>
</tr>
</tbody>
</table>

8. **10% APS**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>1g</td>
</tr>
</tbody>
</table>

Fill up to 10ml with Milli Q water; Stored at -20°C

9. **10X Tris Buffer Saline (TBS)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>30g</td>
</tr>
<tr>
<td>NaCl</td>
<td>80g</td>
</tr>
<tr>
<td>KCl</td>
<td>2g</td>
</tr>
</tbody>
</table>

Fill up to 1L with Milli Q water; Adjusted pH to 7.4 and stored at RT
### 10. 1X TBS-T

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween-20</td>
<td>0.5ml</td>
</tr>
<tr>
<td>10X TBS</td>
<td>50ml</td>
</tr>
</tbody>
</table>

Fill up to 500ml with Milli Q water; Stored at RT

### 11. 0.135 mM NaH$_2$PO$_4$ solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH$_2$PO$_4$•H$_2$O</td>
<td>18.63g</td>
</tr>
</tbody>
</table>

Fill up to 1L with Milli Q water; Stored at RT

### 12. 0.135 mM Na$_2$HPO$_4$ solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$•2H$_2$O</td>
<td>24.03g</td>
</tr>
</tbody>
</table>

Fill up to 1L with Milli Q water; Stored at RT

### 13. 0.135 mM Sodium phosphate buffer (pH 6.2)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.135 mM NaH$_2$PO$_4$ solution</td>
<td>81.5ml</td>
</tr>
<tr>
<td>0.135 mM Na$_2$HPO$_4$ solution</td>
<td>18.5ml</td>
</tr>
</tbody>
</table>

### 14. 0.135 mM Sodium phosphate buffer (pH 7.0)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.135 mM NaH$_2$PO$_4$ solution</td>
<td>39.0ml</td>
</tr>
<tr>
<td>0.135 mM Na$_2$HPO$_4$ solution</td>
<td>61.0ml</td>
</tr>
</tbody>
</table>

### 15. 6X sample buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X Tris-HCl, pH 6.8</td>
<td>7ml</td>
</tr>
<tr>
<td>Glycine</td>
<td>3ml</td>
</tr>
<tr>
<td>SDS</td>
<td>1g</td>
</tr>
<tr>
<td>DTT</td>
<td>0.93g</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>1.2mg</td>
</tr>
</tbody>
</table>

Stored at -80°C
CURRICULUM VITAE

Academic qualifications of the thesis author, Mr. ZHANG Shi Qing:

- Received the degree of Bachelor of Chinese Medicine from Southern Medicine University, July 2009.

- Received the degree of Master of Chinese Medicine from Southern Medicine University, July 2012.

August 2016