Effect of Isothiocyanates and Selenium on Antioxidant Enzyme Expression and DNA Methylation in Colon Cancer Cells *in vitro*

A thesis submitted to the University of East Anglia in accordance with the requirements of the Degree of Doctor of Philosophy

By

Lawrence Barrera

School of Medicine, Health Policy and Practice

University of East Anglia

November 2010

“I certify that the work contained in the thesis submitted by me for the degree of PhD is my own original work, except where due reference is made to other authors, and has not previously been submitted by me for a degree at this or any other university”

Signed

Lawrence Barrera

Acknowledgment

I would like to express my deepest and most sincere gratitude to my UEA PhD supervisors, Yongping Bao and Aedin Cassidy, and IFR PhD supervisors, Nigel Belshaw and Ian Johnson for their expert guidance, remarkable support and instructive comments at every stage of the thesis process.

I am grateful to Faculty of Health at UEA, the Institute of Food Research in Norwich, UK and the Overseas Research Students Awards Scheme (ORSAS), UK for providing me with such a wonderful studentship and financial support to undertake my PhD studies and made my dreams come true.

I am deeply indebted to Michiel Adriaens from the University of Maastricht, Department of Bioinformatics, BiGCaT, who kindly performed the statistical analysis of the CpG island microarray developed during this project. Also thanks to Ruan Elliott for his assistance and technical advice provided during the execution of the microarray experiments.

Completing a PhD is without doubt a marathon experience, and I could not have been able to complete it without the aid and advice of countless people over the past 3 years. I warmly thank Wei Wang for her valuable advice, training and friendly help throughout my PhD. I also thank my lab mates Gary Woodward, Wouter Hendrickx, Julie Decock, Lina Mastrangelo, Eirini Kelaiditi and Ana Wawer at UEA for their help at the bench and funny times lived during this journey. I am also grateful to Wing Leung, Carol Connor and Giles Elliot at IFR for sharing their knowledge I could rely on during experimental set up. I also wish to thank Stuart Rushworth from the School of Pharmacy at UEA for providing me with siRNA *Nrf2* transfection reagents and advice to carry out this part of the work.

I am grateful to the members of the postgraduate office within the Faculty of Health, specially Gilly Potter and Sonia Fagan for their continuous help and exceptional administrative support when required.

I thank my friends Alexander Bueno, Melissa Vieira and Alfredo Ramirez for their friendship and for helping me to decrease my stress levels by having a good laugh when I needed it most. I also thank my sister in law Claudia Sanchez for her kind support.

Lastly, but certainly not least, I would like to thank my parents and grandparents for all their love, prayers and encouragement provided to pursuit my dreams, and for the presence of my loving, caring and patient wife Paula Sanchez whose faithful support throughout my studies has been priceless, you have been there at every stage of my PhD, this thesis is dedicated to you.

Abstract

There is growing evidence that diet, lifestyle, epigenetic changes, genetic mutations and chronic inflammation are main risk factors for colon carcinogenesis. In particular, reactive oxygen and nitrogen species play a significant role in the pathogenesis of several diseases of the gastrointestinal tract, including colon cancer. Also, abnormalities in cytosine methylation such as global hypomethylation and regional promoter hypermethylation are recognised as hallmarks of gastrointestinal neoplasia. From a dietary point of view, two key components are widely believed to exert cancer chemopreventive effects: isothiocyanates (ITCs), present in cruciferous vegetables, and selenium (Se), which are an integral part of many selenoproteins. Therefore, this investigation examined the effect of the ITCs sulforaphane or iberin, either individually or in combination with selenium in the form of selenite or Se-methylselenocysteine, on the expression of antioxidant selenoenzymes thioredoxin reductase-1 (TrxR1) and gastrointestinal glutathione peroxidise (GI-GPx) in an *in vitro* model using Caco-2 cells. The results suggest that the simultaneous addition of ITCs+Se induced TrxR1 and GI-GPx expression more than either compound alone through an Nrf2-dependent mechanism. In addition, a single and double TrxR1/GI-GPx knockdown approach demonstrated that both selenoproteins are responsible for a synergistic protection against H_2O_2–induced cell death when cells are co-treated with ITC+Se. Furthermore, the impact of ITCs and Se on DNA methylation, specifically examining factors modulating gene-specific (*p16* ^{INK4A}, *ESR1, APC, HPP1, MGMT*) and global (LINE-1) methylation, in addition to *DNMT* expression, were examined in Caco-2 and HCT116 cells. However, none of the compounds assessed influenced the methylation status of the genes studied. Taken together, these data shed new light on the relevance of combining ITC and Se to enhance antioxidant defense mechanisms and characterise further their potential role as chemopreventive agents in colon cancer.

List of Abbreviations

Abbreviation	Description
ACF	Aberrant crypt foci
AICR	Association for International Cancer Research
AITC	Allyl Isothiocyanates
APC	Adenomatous polyposis coli
ARE	Antioxidant responsive element
BITC	Benzyl isothiocyanates
BSA	Bovine serum albumin
COBRA	Combined bisulphite restriction analysis
CTCF	CCCTC-binding factor
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyltransferases
EGCG	Epigallocatechin 3-galleate
ERK	Extracellular-signal-regulated kinase
ESR1	Oestrogen receptor α
FDR	False discovery rate
GI-GPx	Gastrointestinal glutathione peroxidise
GPx1	Cytosolic glutathione peroxidise
GPx3	Plasma glutathione peroxidise
GPx4	Phospholipid hydroperoxide glutathione peroxidise
GSH	Glutathione

GSs	Glucosinolates
GSs	Glucosinolates
GST	Glutathione S-transferase
GSTM1	Glutathione S-transferase mu 1
GSTT1	Glutathione S-transferase theta 1
H_2O_2	Hydrogen peroxide
HAT	Histone acetyltransferase
HCA	Heterocyclic amines
HDAC	Histone deacetylase
HPP1	Hyperplastic polyposis protein 1
IB	Iberin
ITCs	Isothiocyanates
JNK	C-Jun NH2-terminal kinase;
Keap1	Kelch-like-ECH-associated protein 1
LINE-1	Long interspersed nuclear element-1
M.SssI	CpG Methyltransferase
MAPKs	Mitogen-activated protein kinases
MeDIP	Methyl-DNA immunoprecipitation
MGMT	O^6-methylguanine-DNA methyltransferase
MSP	Methylation-specific PCR
MSRE	Methylation-sensitive restriction enzymes
NQO1	NAD(P)H quinone oxidoreductase 1
Nrf2	NF-E2-related factor 2

PEITC	Phenethyl isothiocyanates
PHI	Phenylhexyl isothiocyanate
PI3K	Phosphatidylinositol 3-kinase
p-XSC	Phenylenebis-(methylene)-selenocyanate
QMSP	Quantitative methylation-specific PCR
ROS	Reactive oxygen species
ROSN	Reactive oxygen and nitrogen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Se	Selenium
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence element
SelP	Plasma selenoprotein P
SeMet	Selenomethionine
SeMSC	Se-methylselenocysteine
SFN	Sulforaphane
SNPs	Single nucleotide polymorphisms
SSC	Saline-sodium citrate
Trx	Thioredoxin
TrxR1	Thioredoxin reductase-1
WCRF	World Cancer Research Fund International

List of Contents

Acknowledgment.....III
Abstract.....V
List of Abbreviations.....VI
List of Tables..... XV
List of Figures.....XVII

CHAPTER 1:General Introduction 1

1.1 Nutrition and Cancer.....2
1.2 Cruciferous Vegetables and Glucosinolates.....4
1.2.1 Glucosinolate Metabolism 4
1.2.2 Isothiocyanate Metabolism.....6
1.3 Molecular Basis of the Chemoprotective Effects of Isothiocyanates 7
1.3.1 Inhibition of Phase I Enzymes 8
1.3.2 Phase II metabolism.....9
1.3.2.1 Transcriptional Regulation of Phase II Enzymes.....12
1.3.3 Cell Proliferation, Apoptosis and Cell Cycle Arrest Mediated by Isothiocyanates.....14
1.4 Human Cell and Tissue Exposure to Isothiocyanates.....16
1.5 Selenium and Cancer Prevention 18
1.5.1 Selenoprotein Biosynthesis.....20
1.5.2 Metabolism of Different Dietary Selenium Compounds 21
1.5.3 Antioxidant Effects of Selenium.....23
1.5.3.1 Glutathione Peroxidases.....24
1.5.3.2 Thioredoxin Reductases.....25
1.6 Colorectal Carcinogenesis.....28
1.6.1 Caco-2 Cell Line as an *in vitro* Model to Study Colorectal Cancer 31
1.7 Epigenetics.....32
1.7.1 DNA Methylation 32
1.7.1.1 DNA Methyltransferases.....34

1.7.1.2 Repression of Tumour Suppressor Genes 35
1.8 Diet and Epigenetics 38
1.9 Proposed Investigation 41

CHAPTER 2: Modulation of the Expression of Antioxidant Enzymes TrxR1 and GI-GPx by Isothiocyanates and Selenium in Caco-2 Cells 45

2.0 Introduction 46
2.1 Materials And Methods 47
2.1.1 Materials 47
2.1.2 Methods 47
2.1.2.1 Cell Culture 47
2.1.2.2 Cell Viability Assay to Determine the Cytotoxicity of ITCs and Se 48
2.1.2.3 Induction of TrxR1 and GI-GPx by Isothiocyanates and Selenium 49
2.1.2.3.1 Cell Culture 49
2.1.2.3.2 RNA Isolation 50
2.1.2.3.3 Reverse Transcription 50
2.1.2.3.4 Real-Time PCR 50
2.1.2.3.5 Western Blot Analysis 52
2.1.2.4 Role of Nrf2 in Isothiocyanate- and/or Selenium-mediated Antioxidant Enzyme Expression 54
2.1.2.4.1 *Nrf2* siRNA Interference Assay 54
2.1.2.4.2 Quantification of Nrf2 by Real-Time PCR 56
2.1.2.4.3 Nuclear and Cytoplasmic Protein Extraction 57
2.1.2.5 Effects of Hydrogen Peroxide on Caco-2 Cells Pre-treated with Isothiocyanates and/or Selenium Compounds. 58
2.1.2.5.1 Cytotoxicity of Hydrogen Peroxide 58
2.1.2.5.2 ITC- and/or Se-mediated Cytoprotection in Caco-2 Cells 59

2.1.2.6 Role of *TrxR1* and *GI-GPx* in the ITC- and/or Se-mediated Cytoprotection in Caco-2 cells.....59
2.1.2.6.1 TrxR1/GI-GPx siRNA Optimisation Assay.....59
2.1.2.6.2 Cell Viability Effects of *TrxR1* and/or *GI-GPx* Knockdown in Caco-2 cells.....60
2.1.3 Statistical Methods.....61
2.2 Results and Discussion.....62
2.2.1 Effect of Isothiocyanates and Selenium on Cell Survival.....62
2.2.2 Up-regulation of TrxR1 and GI-GPx by Isothiocyanates and Selenium Compounds.....68
2.2.3 Up-regulation of Nrf2-mediated Antioxidant Enzyme Induction.....79
2.2.3.1 Nrf2 Transcriptional and translational Expression Pattern in ITCs and/or Selenium Treated Caco-2 Cells.....79
2.2.3.2 *Nrf2* siRNA Interference Assay.....83
2.2.4 Effects of Hydrogen Peroxide on Caco-2 Cells Pre-treated with Sulforaphane and/or Selenite.....85
2.2.4.1 Cytotoxicity of Hydrogen Peroxide.....85
2.2.4.2 ITCs and/or Se-mediated Cytoprotection in Caco-2 Cells.....86
2.2.5 Role of TrxR1 and GI-GPx in the Synergism between ITC and Se-mediated Cytoprotection in Caco-2 Cells.....88
2.2.5.1 *TrxR1*/*GI-GPx* siRNA Optimisation Assay.....88
2.2.5.2 Effect of *TrxR1* and/or *GI-GPx* Knockdown on H_2O_2- Induced Damage in Caco-2 Cells Pre-treated with SFN+Selenite.....89

CHAPTER 3: Impact of Isothiocyanates and Selenium on Global and Gene-specific Methylation Patterns and Effects on DNA Methyltransferase Gene Expression in Colon Cancer Cells *in vitro*.....94

3.0 Introduction.....95
3.0.1 Analytical Methods Used to Quantify DNA Methylation.....96
3.1 Materials and Methods.....99
3.1.1 Materials.....99

3.1.2 Methods.....99
3.1.2.1 Cell Culture and Treatments.....99
3.1.2.2 Genomic DNA and RNA Isolation.....101
3.1.2.3 Bisulphite Treatment of Genomic DNA.....102
3.1.2.3.1 Standard Bisulphite Treatment.....102
3.1.2.3.2 Bisulphite Treatment: High-throughput.....103
3.1.2.4 Initial PCR Amplification of CpG Islands.....103
3.1.2.5 Quantitative Methylation-Specific PCR (QMSP).....104
3.1.2.6 Global Methylation.....105
3.1.2.7 Standard Curve: Quantification of Methylated Alleles.....106
3.1.2.8 Preparation of Controls for DNA Methylation.....107
3.1.2.8.1 Non-cancerous Tissue.....107
3.1.2.8.2 5-Aza-2'-Deoxycytidine Treatment.....107
3.1.2.9 Quantification of DNA Methyltransferases by Real-Time PCR.....108
3.1.2.10 Statistics.....109
3.2 Results and Discussion.....110
3.2.1 Effect of Iberin and SeMSC on Epigenetic Modifications: A DNA Methylation Screening in Caco-2 cells.....110
3.2.2 DNA Methylation Dynamics Over Time: Influence of Iberin and/or SeMSC on DNA methylation Changes in a Time-Dependent Manner. .112
3.2.3 Effect of Time and Dose of Different Isothiocyanates and Selenium Forms on DNA Methylation and DNA Methyltransferase Expression. .114
3.2.4 DNA Methylation Status of HCT116 Cells.....118

CHAPTER 4: Applications and Challenges of Genome-wide DNA Methylation Analysis using CpG Island Microarrays in Nutrition Studies.....128

4.0 Introduction.....129
4.1 Materials and Methods.....131
4.1.1 Materials.....131
4.1.2 Methods.....131

4.1.2.1 Cell Culture and Treatments 131
4.1.2.2 First Microarray Protocol 131
4.1.2.2.1 Amplicon Preparation 132
4.1.2.2.1.1 *Mse*I Restriction 132
4.1.2.2.1.2 Annealing of Oligonucleotides 133
4.1.2.2.1.3 Linker Ligation to MseI-Digested DNA 133
4.1.2.2.1.4 Digestion of Linker-Ligated DNA ± McrBC 134
4.1.2.2.2 Labelling and Hybridization 135
4.1.2.2.2.1 Aminoallyl-dUTP Incorporation 135
4.1.2.2.2.2 Cy Dye Coupling 136
4.1.2.2.3 Microarray Hybridization 136
4.1.2.2.4 Washing and Scanning 138
4.1.2.2.5 Data Processing 138
4.1.2.2.6 Data Normalization 139
4.1.2.2.7 Validation of Detected Methylation Differences 140
4.1.2.2.7.1 COBRA 140
4.1.2.2.7.1.1 COBRA PCR Primer Design 140
4.1.2.2.7.1.2 PCR Annealing Temperature Optimization 141
4.1.2.2.7.1.3 Outline of the COBRA Protocol 141
4.1.2.2.7.2 Methylation-Sensitive Restriction Enzymes (MSRE) 142
4.1.2.2.7.2.1 PCR Primer Design for Real-Time PCR Following MSRE Digestion of Genomic DNA 143
4.1.2.2.7.2.2 Outline of the MSRE Protocol 144
4.1.2.3 Second Microarray Protocol 145
4.1.2.3.1 Sample Preparation 146
4.1.2.3.2 Amplicon Preparation 146
4.1.2.3.2.1 DNA Fractionation 146
4.1.2.3.2.2 McrBC Digestion 148
4.2 Results and Discussion 150
4.2.1 First Microarray Protocol Validation 150
4.2.2 Second Microarray 155

CHAPTER 5: Discussion & Future Perspectives.....161

5.0 General Discussion 162
5.1 Future Perspectives 172

REFERENCES.....175

List of Tables

CHAPTER 1

Table 1.1 Glucosinolates and chemical structures of isothiocyanates found in commonly eaten cruciferous vegetables 5
Table 1.2 Genes induced by Nrf2.....13
Table 1.3 Pathways Disrupted by Gene-Promoter Hypermethylation and Associated Gene Silencing in Cancer. 36

CHAPTER 2

Table 2.1 Primers and Probes Sequences Used for RT- PCR 52
Table 2.2 Primers and Probes Sequences Used for RT-PCR 56
Table 2.3 IC_{50} values of sulforaphane and iberin obtained after treating Caco-2 cells from 24 to 96 h.....63
Table 2.4 IC_{50} values of selenite and SeMSC obtained after treating Caco-2 cells from 24 to 96 h.....66

CHAPTER 3

Table 3.1 Primer Sequences and Annealing Temperatures for Initial PCR and QMSP.....104
Table 3.2 Primers and Probes Sequences Used for RT-PCR 109

CHAPTER 4

Table 4.1 Thermocycler Programs for Annealing Complementary Oligonucleotides 133
Table 4.2 Primer Sequences and Annealing Temperatures for the Analysis of Methylation at the Indicated CpG islands.....142
Table 4.3 Primer Sequences and Annealing Temperatures used for Methylation-sensitive RT-PCR.....144

Table 4.4 Methylated and Non-methylated DNA fraction used to generate specific DNA methylation profiles 146
Table 4.5 Settings applied to the HydroShear machine to optimize DNA fragment size 147
Table 4.6 Results from IL4R Band Quantification 151
Table 4.7 Candidates Genes from the Hypermethylated Group.....152
Table 4.8 Candidates Genes from the Hypomethylated Group.....152
Table 4.9 Candidates Genes Selected for Validation and Methylation Changes Obtained Using Microarray and MSRE.....154

List of Figures

CHAPTER 1

Figure 1.1 Classification of dietary phytochemicals.....4
Figure 1.2 General scheme of the hydrolysis of glucosinolates to isothiocyanates.....5
Figure 1.3 Isothiocyanates Metabolism.....6
Figure 1.4 Role of oxidative stress and antioxidant systems in a simplified scheme of carcinogenesis.....10
Figure 1.5 Mechanisms of isothiocyanates-induced apoptosis of tumour cells.....15
Figure 1.6 Selenoprotein synthesis.....21
Figure 1.7 Pathways of selenium metabolism.....23
Figure 1.8 Functions of the thioredoxin system.....27
Figure 1.9 An adaptation of the Vogelstein adenoma-carcinoma sequence.....30
Figure 1.10 Biochemistry of DNA methylation.....33
Figure 1.11 Distribution of CpG islands in the human genome.....34
Figure 1.12 Folate and one-carbon metabolism: regulation of DNA synthesis, repair and methylation.....39

CHAPTER 2

Figure 2.1 Effect of sulforaphane and iberin on Caco-2 cells viability.....62
Figure 2.2 Effect of selenite and SeMSC on Caco-2 cells viability.....64
Figure 2.3 Time course of isothiocyanates-induced TrxR1 and GI-GPx mRNA in Caco-2 cells.....68
Figure 2.4 Dose dependent effects of isothiocyanates on TrxR1 and GI-GPx mRNA expression in Caco-2 cells 70
Figure 2.5 Effect of ITCs and/or Se supplementation on TrxR1 mRNA expression in Caco-2 cells.....71

Figure 2.6 Effect of ITCs and/or Se supplementation on GI-GPx mRNA expression in Caco-2 cells.....72
Figure 2.7 Differential levels of TrxR1 protein expression in Caco-2 cells following supplementation of ITCs and/or Se for 8, 24 and 48 h..... 73
Figure 2.8 Differential levels of GI-GPx protein expression in Caco-2 cells following supplementation of ITCs and/or Se for 8, 24 and 48 h.....74
Figure 2.9 Effect of ITCs and/or Se supplementation on Nrf2 mRNA in Caco-2 cells 81
Figure 2.10 Representative images of Nrf2 immunoblots with cytosolic and nuclear fractions derived from control, iberin- or iberin+SeMSC-exposed Caco-2 cells. 82
Figure 2.11 Effect of ITCs and/or selenite treatment on TrxR1 and GI-GPx expression in *Nrf2* suppressed Caco-2 cells. 84
Figure 2.12 Cytotoxic effect of H_2O_2 on Caco-2 cells untreated or pre-treated with selenite. 85
Figure 2.13 Effect of ITCs and/or selenium on cell viability in Caco-2 cells treated with hydrogen peroxide. 86
Figure 2.14 siRNA knockdown efficiency of *TrxR1* and/or *GI-GPx* expression in Caco-2 cells.....88
Figure 2.15 Cell viability effects of *TrxR1* and/or *GI-GPx* knockdown in Caco-2 cells.....90

CHAPTER 3

Figure 3.1 Schematic flowchart showing DNA methylation analysis by MethyLight technology.....97
Figure 3.2 Schematic design of the schedule followed to treat cells to study DNA methylation changes in colon cancer cells.....101
Figure 3.3 Chemical structures of Cytidine and 5-Aza-2'-deoxycytidine, highlighting their main structural difference.....108

Figure 3.4 DNA methylation status of *p16* ^{INK4A}, *ESR1*, *HPP1*, *APC*; and MGMT CpG islands in Caco-2 cells exposed to DMSO (control), 6 µM of iberin and/or 200 nM of SeMSC for 4 days 111
Figure 3.5 DNA methylation status of *p16* ^{INK4A}, *ESR1*, and LINE-1 in Caco-2 cells exposed to DMSO (control), 6 µM of iberin and/or 200 nM of SeMSC for 4, 8 and 12 days.....113
Figure 3.6 DNA methylation status of *p16* ^{INK4A}, *ESR1* in Caco-2 cells treated with SeMSC, selenite, iberin, SFN, SFN+SeMSC and 5-Aza-2'-D in a time- and dose-dependent manner.....115
Figure 3.7 DNA methylation status of LINE-1 in Caco-2 cells treated with SeMSC, selenite, iberin, SFN, SFN+SeMSC and 5-Aza-2'-D in a time- and dose-dependent manner.....116
Figure 3.8 Effect of SeMSC, selenite, iberin, SFN, SFN+SeMSC and 5-Aza-2'-D on DNMT1, DNMT3A, DNMT3B mRNA in Caco-2 cells.....117
Figure 3.9 DNA methylation status of *p16* ^{INK4A}, *ESR1* and LINE-1 in HCT116 cells treated with SeMSC, selenite, iberin, SFN, and its combination.....120
Figure 3.10 Effect of SeMSC, selenite, iberin, SFN and its combination on DNMT1, DNMT3A, DNMT3B mRNA in HCT116 cells.....122

CHAPTER 4

Figure 4.1 Schematic flowchart for amplicon preparation in CpG island microarray.....137
Figure 4.2 HydroShear DNA shearing device used to fractionate the DNA into smaller fragments.....147
Figure 4.3 DNA fragment sizes generates by the HydroShear DNA shearing device 148
Figure 4.4 DNA methylation fractions (0, 30, 60, 100%) McrBC digested and mock-digested resolved on a 1% agarose gel.....149
Figure 4.5 Chart displaying numbers of genes with statistically significant epigenetic changes per treatment group.....150
Figure 4.6 COBRA assay showing bands obtained after enzyme digestion..151

Figure 4.7 Histogram of signals with model overlay from groups with known percentage of methylation.....156
Figure 4.8 Graphs illustrating measured percentage of methylation (methylation scores) for various CpG islands and their expected percentage of methylation, and scatter plot of 150 random CpG islands depicting the inability of the methylation score model and/or microarray platform to predict expected percentages of methylation adequately.....157

1

General Introduction

1.1 Nutrition and Cancer

Diet and lifestyle play an important role in cancer aetiology and it has been estimated that specific dietary patterns and constituents are key components of the environmental influences that may contribute to the development of 1/3 of human cancers (WCRF/AICR 2007). During the early 90s expectations arose as increased consumption of fruit and vegetables showed evidence for protective effects against cancer. At this time most of the epidemiologic literature on the relationship between vegetables and fruit consumption and human cancer, at a variety of sites, was associated consistently with a reduced risk of cancers (Steinmetz and Potter 1991). Rapidly a number of government programmes were created in the United States and Europe to increase fruit and vegetable consumption. These include the 'Five-A-Day for better health' campaign program to encourage people to eat fruit and vegetables –at least five servings a day- to reduce the risk of cancer and other diseases. However, the evidence for a large preventive effect was based primarily on data from case-control studies, which are susceptible to recall bias (Holmberg et al. 1996). Then in the late 1990s, when the results of prospective cohort studies of diet and cancer began to emerge, their outcome did not confirm the strong inverse association found in most case control studies. All these studies were summarised in a comprehensive report by the World Cancer Research Fund (WCRF/AICR 2007) where most of the outcomes obtained from the data analyses reversed previous conclusions concerning a strong benefit of fruit and vegetable consumption for protection at various cancer sites published 10 years previously (WCRF/AICR 1997).

These conclusions were recently confirmed in a prospective analysis of nearly 400,000 men and women who developed approximately 30,000 cancers at all sites combined over nearly 9 years of follow-up from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort to assess relationships between intake of fruits and vegetables and cancer risk. After accounting for measurement error, a very weak but statistically significant inverse association

was seen, showing that in the best case scenario an increment of 200 g of fruit and vegetables each day (which corresponds to an extra two portions) could prevent 2.6% of cancers in men and 2.3% of cases in women (Boffetta et al. 2010).

However, a very weak association between fruits and vegetables and risk of cancer does not exclude the possibility that a small group of fruit and vegetables, or a specific food compound present in some of these groups, has an important protective effect (Willett 2010). In this respect, the emerging evidence for a variety of potentially-important components present in plant-based foods that possess cancer-preventive properties has stimulated interest in the concept of chemoprevention and particular attention has been focused on particular fruits and vegetables, specifically citrus fruits, dark green vegetables, and cruciferous vegetables, that have been shown to influence various stages in the development of cancer (IARC/WHO 2004).

Several cellular processes appear to be modulated by some food components influencing cancer growth and/or tumour behaviour. These include, but are not limited to, carcinogen metabolism, DNA repair, cell proliferation, cell death, inflammation, differentiation and angiogenesis (Davis and Milner 2007). Some of these associations between higher consumption of fruits and vegetables and reduced risk of cancers in epidemiological and mechanistic studies have been suggested to be as a result of its content of biologically active chemicals, including established nutrients but with greater interest in the bioactive constituents named phytochemicals. These compounds correspond to a wide range of biologically-active secondary plant metabolites that provide plants with colour, flavour and natural protection against pests and have been linked to reduction in the risk of major chronic diseases (Johnson et al. 1994). It is estimated that >5000 individual phytochemicals have been identified in fruit, vegetables and grains, but a large percentage still remain unknown (Liu 2003). They are classified according to their chemical structure and functional characteristics and include various carotenoids, phenolics and organosulfur

compounds within which our group of interest, isothiocyanates, can be found (Figure 1.1).

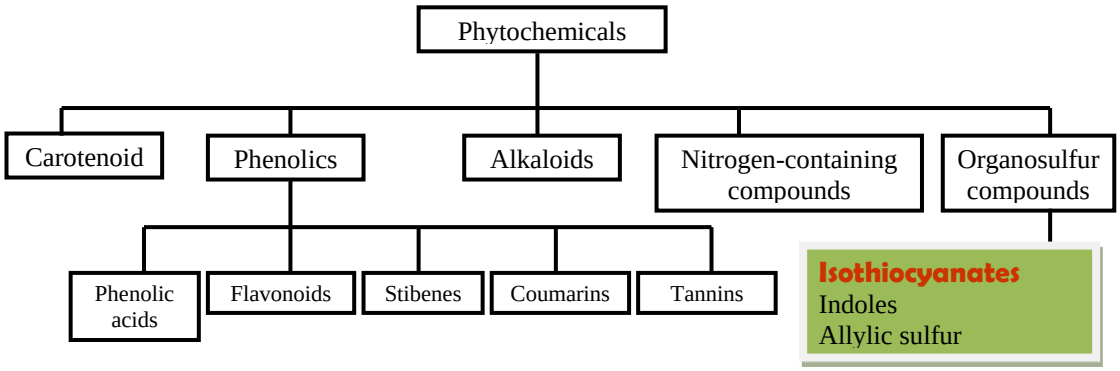


Figure 1.1 Classification of dietary phytochemicals.

1.2 Cruciferous Vegetables and Glucosinolates

Glucosinolates (GSs) are an important group of phytochemicals, present in Brassica vegetables including broccoli, watercress, brussel sprouts, cabbage, Japanese radish and cauliflower. Within the plant, the GS content can vary between and within member of the cruciferous vegetables depending on cultivation environment and genotype.

1.2.1 Glucosinolate Metabolism

Glucosinolates are relatively biologically inert, but can degrade to a range of bioactive compounds, such as isothiocyanates (ITCs) and indoles, on hydrolysis by the plant-based enzyme myrosinase, but in the human diet the myrosinase in cruciferous vegetables is often heat-inactivated, and in this situation GSs can also be hydrolysed less efficiently by the colonic microflora (Fahey et al. 2001) (Figure 1.2). The glucosinolate molecule comprises two parts: a common glycone moiety and a variable aglycone side chain derived from amino acids. While over 120 GSs have been identified in various plants (Fahey et al. 2001), cruciferous vegetables are the principal dietary source of ITCs, but the types of crucifers frequently consumed by humans are mostly limited to the ones mentioned above and a few others.

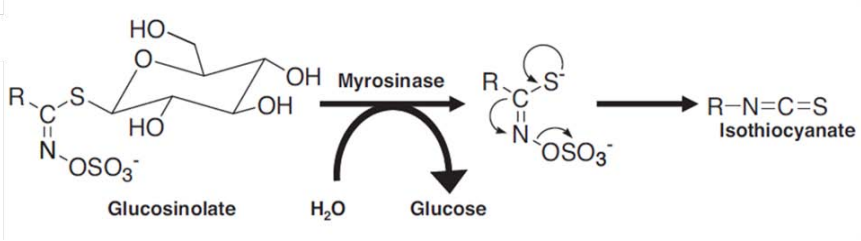
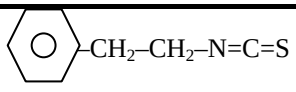


Figure 1.2 General scheme of the hydrolysis of glucosinolates to isothiocyanates. Adapted from (Nakamura and Miyoshi 2010)

Examples of common crucifers that are particularly rich in certain ITCs include: mustard and horseradish, which are rich in allyl-ITC (AITC) found in the form of its corresponding GS sinigrin; watercress, which is rich in phenethyl-ITC (PEITC), found as gluconasturtiin; broccoli and broccoli sprouts, rich in the GS glucoraphanin, which gives rise to one of the most well-characterised ITCs sulforaphane (SFN). Many other ITCs that are present in lower quantities also may contribute to the anti-carcinogenic properties of crucifers (Table 1.1).

Table 1.1 Glucosinolates and chemical structures of isothiocyanates found in commonly eaten cruciferous vegetables

Glucosinolates	Associated Isothiocyanates	Chemical Structure of Isothiocyanates and Plant Source
Glucoraphanin	Sulforaphane	$CH_3–S–CH_2–CH_2–CH_2–CH_2–N=C=S$ || O Broccoli, Brussels sprouts
Glucoiberin	Iberin	$CH_3–S–CH_2–CH_2–CH_2–N=C=S$ || O Broccoli, some Brussels sprouts and cabbages
Glucoerucin	Erucin	$CH_3–S–CH_2–CH_2–CH_2–\ CH_2–N=C=S$ Rockets
Sinigrin	Allyl-ITC (AITC)	$CH_2=CH–CH_2–N=C=S$ Mustard and horseradish
Gluconasturtiin	Phenethyl-ITC (PEITC)	 $–CH_2–CH_2–N=C=S$ Watercress, radishes, turnips

1.2.2 Isothiocyanate Metabolism

Studies in both humans and experimental animals have demonstrated that exposure of cells to ITCs can lead to a rapid high intracellular accumulation. Reduced glutathione (GSH), the most abundant thiol-carrying molecule in a cell, is known to be primarily responsible for the conjugation of ITCs, which takes place spontaneously but is further enhanced by glutathione *S*-transferase (GST) resulting in the formation of dithiocarbamates through the mercapturic pathway with the excretion of N-acetylcysteine in urine (Zhang and Callaway 2002) (Figure 1.3).

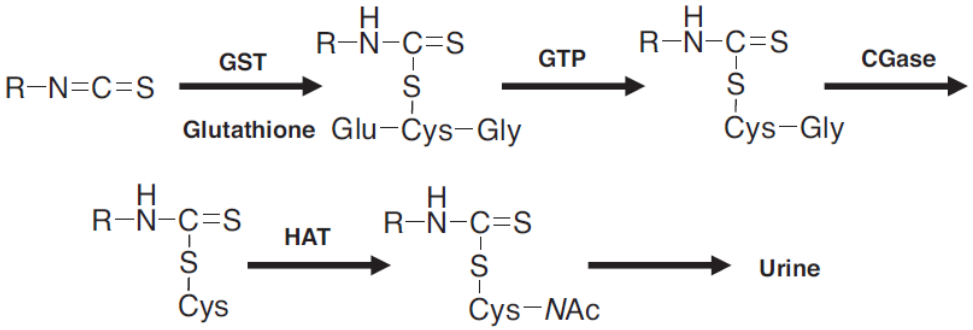


Figure 1.3 Isothiocyanates (R–N=C=S) are conjugated to glutathione by glutathione S-transferase (GST), metabolized sequentially by γ-glutamyltranspeptidase (*GTP*), cysteinylglycinase (*CGase*) and histone acetyltransferase (HAT) to form, ultimately, mercapturic acid NAc, N-acetylcysteine for urine excretion. Adapted from (Nakamura and Miyoshi 2010)

Glutathione is known to maintain the cellular oxidation-reduction balance and protects cells against free radical species. Despite clear evidence that ITCs stimulate cellular antioxidant proteins that protect cells against oxidative damage and carcinogens (see section 1.3.2 below), it has also become increasingly apparent that they induce cellular stress as conjugation with glutathione (GSH) render the cells susceptible to oxidative stress and stress-induced damage (Clarke et al. 2008). Moreover, conjugated ITC-GSH have been shown to be removed by membrane transporters including multidrug resistant associated protein 1 (MRP-1) and P-glycoprotein-1 (Pgp-1) and are transferred back to the extracellular medium (Zhang and Callaway 2002).

These conjugates are thought to release more ITCs for re-accumulation in cells which results in further GSH reduction that has been proposed to alter normal cellular functions and promote the induction of oxidative stress associated with ITC-induced apoptosis (Kim et al. 2003).

1.3 Molecular Basis of the Chemoprotective Effects of Isothiocyanates

Understanding the chemoprotective mechanisms of isothiocyanates is important not only because these natural compounds block the formation of a wide variety of carcinogen-induced tumours in rodents, but also because isothiocyanates and their glucosinolate precursors are widely available in human dietary plants and are consumed in significant quantities (Zhang and Talalay 1994).

Consumption of cruciferous vegetables has been more strongly associated with cancer protection than vegetable consumption in both animal models and from available prospective cohort data. Verhoeven et al. (1996) reviewed the evidence for *Brassica* consumption and cancer risk, and reported that 67% of all studies showed an inverse association between total *Brassica* vegetable intake and risk of cancer at various sites (Verhoeven et al. 1996) and a wide range of studies in humans, animals and *in vitro* has confirmed this finding (IARC/WHO 2004). For instance, cruciferous vegetables have been found to reduce morphological markers of colon cancer risk in dimethylhydrazine treated rats (Arikawa and Gallaher 2008). Also in Japan, a public health centre-based prospective study carried out to investigate associations between fruit and vegetable consumption and risk of oesophageal squamous cell carcinoma (SCC), identified that only cruciferous vegetables were associated with a significant decrease in the risk of oesophageal SCC (Yamaji et al. 2008). Consistent with this study, a case-control study recently found that fruit and vegetable intake was unassociated with lung cancer risk among smokers, but increased cruciferous vegetable intake was significantly associated with

reduction of lung cancer risk among smokers (Tang et al. 2010). Numerous studies suggest that phytochemicals in *Brassica* vegetables are responsible for their chemo-protective effects, and among them, glucosinolates are believed to be responsible for this anti-carcinogenic action (Holst and Williamson 2004). As mentioned above, following cell disruption they are hydrolysed forming a group of biologically active compounds known as isothiocyanates. These breakdown products influence the process of carcinogenesis partly by modulation of phase I and II enzymes, induction of apoptosis and cell cycle arrest (Johnson 2002; Zhang 2004). These properties of SFN and other ITCs have led to the view that they are important cancer chemopreventive agents capable of inhibiting multiple steps in the carcinogenesis process (Zhang et al. 2006).

1.3.1 Inhibition of Phase I Enzymes

Many of the dietary and environmental carcinogens to which humans are exposed are subjected to enzymatic transformation once they enter the human body. This process consists of the addition of oxygen, which causes the chemical molecule to become more hydrophilic and consequently more readily excreted. Such transformation is referred to as Phase I metabolism and is catalysed by cytochrome P450 enzymes (CYPs). Consequently, pro-carcinogens are usually converted into highly reactive intermediates that can react with critical macromolecules such as DNA, RNA and protein, resulting in adducts. DNA adducts that persist unrepaired can generate mutations in critical genes such as oncogenes and tumour suppressor genes (Hecht 1999; Juge et al. 2007). Therefore, the inhibition of phase I enzymes is thought to be a preventive measure against chemically induced carcinogenesis. A dose-dependent inhibition of CYP1A1 and CYP2B1/2 by sulforaphane was observed in rat hepatocytes, and also the expression of CYP3A4, the major CYP in human liver, was markedly decreased at both mRNA and activity levels (Maheo et al. 1997). Additionally, treatment of cells with ITCs has been shown to reduce adducts produced from exposure to heterocyclic amines

(HCAs) derived from cooked meat, which has been implicated in the aetiology of certain human cancers including colon, prostate and breast cancer (Takashi et al. 2004). In this respect, SFN has been shown to decrease the number of adducts caused by one of the most abundant types of HCA formed in cooked meat and fish 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) by ~40%, in a dose-dependent manner in human HepG2 cells pre-treated with 1-10 μM SFN followed by 10 nM of PhIP. However, there was no decrease in DNA adduct formation with post-treatment with SFN, suggesting that SFN had no effect on PhIP DNA-adduct repair, but rather prevented PhIP interactions with DNA (Bacon et al. 2003).

1.3.2 Phase II metabolism

Blocking carcinogen metabolic activation and promoting carcinogen detoxification are two ways to decrease carcinogenesis. As already discussed in the preceding section, mammalian cells have evolved mechanisms for protection that convert carcinogens to inactive metabolites that are readily excreted from the body. Early evidence suggesting that increased consumption of fruit and vegetable was associated with reduced risk of developing cancer was initially thought to be due to the presence of phase II enzyme inducers in edible plants. However, when the inducer potencies of a variety of frequently consumed plants were measured, cruciferous plants were shown to rank higher as phase II enzyme inducers (Prochaska et al. 1992).

Much of the evidence points out that a decrease in the expression of antioxidant enzymes together with an increase in the production of free radical species might render cells susceptible to permanent damage and initiate the sequence of events leading to cancer. On the other hand, elevation of phase II enzymes and other antioxidant systems in specific tissues exposed directly to bioactive food components, such as the colon, may confer cytoprotection against the toxicity of electrophiles and ROS (Talalay et al. 1995). These groups of functionally diverse phase II enzymes include glutathione

transferases (GSTs), UDP-glucuronosyltransferases (UGTs), and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Figure 1.4). The modulation of phase II gene expression and enzyme activity by ITCs has been assessed in a number of cell lines of different origin, the most commonly utilized being derived from liver hepatoma, human HepG2 and mouse Hepa1c1c7. For instance, SFN and its glutathione conjugate was found to increased significantly in a time-dependant manner both UGT1A1 and GSTA1 mRNA levels in HepG2 and HT29 cells (Basten et al. 2002). In another study when mouse Hepa1c17 cells were incubated with 2.5 µM of SFN an increase in NQO1 activity was observed, reaching a maximum induction of 3 fold over control (Matusheski and Jeffery 2001).

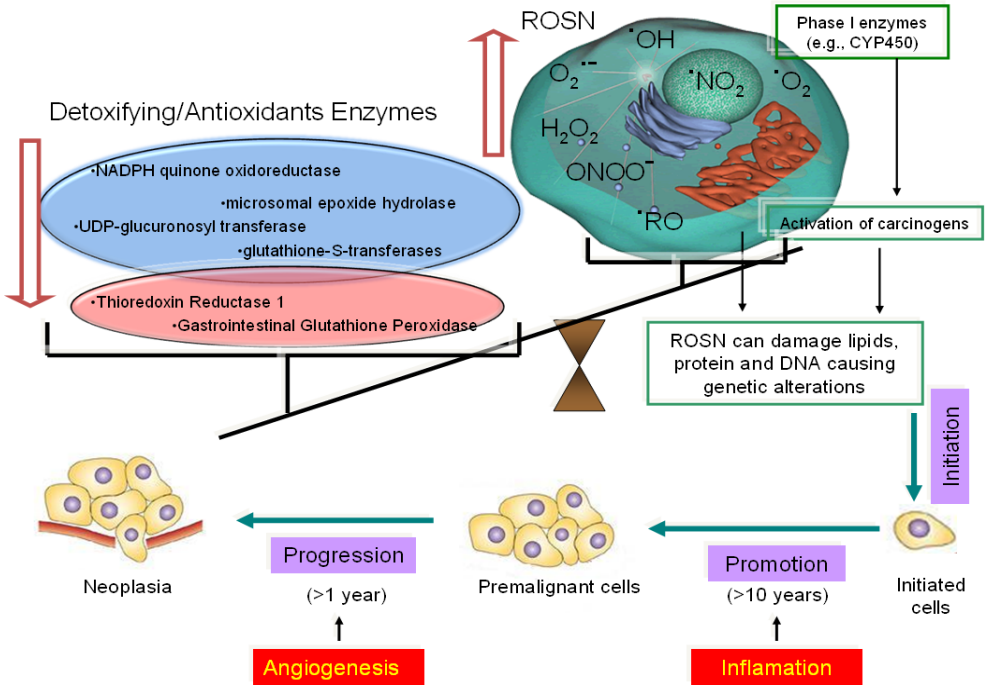


Figure 1.4 Role of oxidative stress and antioxidant systems in a simplified scheme of carcinogenesis. Reactive oxygen and nitrogen species (ROSN) such as the superoxide radical ($O_2^{\bullet -}$), peroxynitrite ($ONOO^-$ or $^{\bullet}NO_2$), hydrogen peroxide (H_2O_2), singlet oxygen ($^{\bullet}O_2$) and the hydroxyl radical ($^{\bullet}OH$) are constantly generated in cells as unwanted by-product of aerobic metabolism and this together with the inflammatory microenvironment of the tissue acts as a predisposing factor to multistage carcinogenesis (Kumar Kundu et al. 2009). Although a low physiologic level of ROSN is scavenged efficiently by the cellular antioxidant defence system an imbalance between the generation of ROSN and cellular antioxidant capacity, as shown above, turns into a state of oxidative stress that contributes to carcinogenesis.

Cell-based models have been used extensively for screening and detection of novel cancer chemopreventive agents from food compounds. However, the induction level and type of phase II enzyme induction vary with different cell lines as reported in a study that used seven widely-adopted cell lines, including Hepa1c1c7, HepG2, MCF7, MDA-MB-231, LNCaP, HeLa and HT-29 where the effects of 25μM of SFN on the enzymatic activity of GST, NQO1, aldo-keto reductase and glutathione reductase were evaluated (Jiang et al. 2003).

This tissue-specific response has been confirmed *in vivo* after analysing the ability of six plant-derived ITCs (allyl-ITC, iberverin, erucin, SFN, iberin and cheirolin) to increase tissue levels of NQO1 and GST in a variety of rat tissues at doses of 40μmol/kg/day. The results revealed different levels of enzyme induction in the duodenum, forestomach and urinary bladder out of 15 tissues analysed, and such responses differed depending on the ITC employed (Munday and Munday 2004). Such difference observed at the cell and tissue level may be explained by the degree at which different ITCs accumulate in cells as demonstrated by Zhang et al (1998), who examined the accumulation of nine ITCs with very different structures at 5 μM each in Hepa 1c1c7 for 24 hours. After measuring two phase II enzymes NQO1 and GST the author demonstrated that those ITCs with an increased level of accumulation in cells induced higher levels of phase II enzymes, demonstrating that these changes were structure-related and influenced by cellular GSH levels (Zhang and Talalay 1998).

Also, extracts of a hybrid between broccoli and a wild relative of broccoli, *Brassica villosa*, resulted in a more than 80-fold increase in the induction of NQO1 in Hepa1c1c7 cells over that with extracts from standard broccoli cultivars. The superior potency was as a result of an increase in glucoraphanin and its greater conversion to SFN (Faulkner et al. 1998; Mithen et al. 2003). The same group recently carried out a human intervention study in healthy volunteers to quantify changes in gene expression in the gastric mucosa following a single meal of standard and high glucosinolate broccoli. The single

meal of super broccoli caused up-regulation of several xenobiotic genes including detoxification enzymes, whereas a single meal of a regular commercial variety did not. The glucosinolate content of the super broccoli meal was 573 µmol SFN, over 3 fold higher than the conventional broccoli meal (170 µmol SFN) (Gasper et al. 2007). In another human study, jejunal perfusion of broccoli extracts (equivalent to ~1.2 g dry weight broccoli) resulted in an induction of GSTA1 and UGT1A1 in exfoliated enterocytes; the changes in gene expression were also confirmed in Caco-2 cells, where sulforaphane was responsible for the induction GSTA1 (3-fold) (Petri et al. 2003).

Since the initiation of many tumours results from damage to DNA by electrophilic carcinogen metabolites or by reactive oxygen and nitrogen species (ROSN), the elevation of enzymes that metabolize xenobiotics may be particularly relevant to the protective effect of cruciferous vegetables to provide protection by increasing the antioxidant capacity of animal cells and their ability to handle oxidative stress (Figure 1.4).

1.3.2.1 Transcriptional Regulation of Phase II Enzymes

As described above the stimulation of phase II enzymes is one of the most important components of cellular defence mechanisms and the ability of ITCs to promote their activation is now understood to be enabled by the Nrf2/ antioxidant response element (ARE) system. However, the list of detoxifying or antioxidant enzymes stimulated by ITCs is not limited only to the phase II group as the Nrf2/ARE signalling pathway targets a variety of enzymes (Table 1.2).

Table 1.2 Genes induced by Nrf2. Adapted from (Brigelius-Flohe and Banning 2006)

Targets for Nrf2
Phase 2 detoxifying enzymes
UDP-glucuronosyl transferase
microsomal epoxide hydrolase
glutathione-S-transferases
NADPH quinone oxidoreductase
Redox-active proteins
heme oxygenase 1
ubiquitin/PKC-j-interacting protein
peroxiredoxin 1
thioredoxin
thioredoxin reductase-1*
dihydrodiol dehydrogenase
cyclooxygenase-2
GSH-related enzymes
γ-glutamyl-cysteine synthetase
cystine/glutamate exchange transport system Xc
gastrointestinal glutathione peroxidase (GI-GPx)*
leukotriene B4 dehydrogenase

* Selenoproteins

Nrf2 (NF-E2-related factor-2) is a member of the NF-E2 family of basic leucine zipper transcription factors (b-zip). Nrf2 protein is found primarily in the cytoplasm and interacts with Keap1 (the Kelch-like ECH-associated protein-1), which recruits an E3 ubiquitin ligase, resulting in Nrf2 ubiquitination and therefore degradation by the proteosome preventing its activation. Inducers, such as ITCs, react with Keap1 leading to loss of its repressor activity and subsequently allowing Nrf2 to undergo nuclear translocation where it binds to the ARE, which is present in the promoter region of genes encoding phase 2 enzymes and enzymes of the antioxidant system and activates their transcription (Thimmulappa et al. 2002; Dinkova-Kostova and Talalay 2008; Eggler et al. 2008). However, apart from its role in the activation of antioxidant and detoxifying enzymes shown in table 2, more recently several studies have indicated a positive role of Nrf2 in cancer tumorigenesis and chemoresistance (Lau et al. 2008).

1.3.3 Cell Proliferation, Apoptosis and Cell Cycle Arrest Mediated by Isothiocyanates

Deregulated proliferation and inhibition of apoptosis are two of the main consequences of tumour development and represent two potentially important mechanisms for dietary chemoprevention (Martin 2006). Survival of all somatic cells requires the continuous input of survival signals to suppress apoptosis. The central machinery responsible for the initiation and the execution of cell death by the cleavage of a variety of intracellular substrates that trigger the cell dissolution process is a family of cysteine and aspartyl proteases termed caspases (Evan and Vousden 2001). Additionally, the progression of the cell cycle through the four phases, G1, S, G2 and M, is regulated by cyclin-dependent kinase (CDK) molecules and cyclins, which drive the cells from one phase to the next. Elucidation of the critical events associated with tumour formation provides the opportunity for dietary intervention to target key events during carcinogenesis particularly by bioactive agents such ITCs. In this scenario, treatment of cells with ITC leads to activation of caspases involved in multiple apoptotic pathways. For instance, in HL60 cells, benzyl-ITC (BITC) and allyl-ITC (AITC) at 10 µM activated caspase 9 (the mitochondria pathway), caspase 8 (the death receptor pathway, DR5) and caspase 12 (the endoplasmic reticulum pathway, ER), with subsequent activation of caspase 3 (Zhang et al. 2003) (Figure 1.5).

The classical hallmark of apoptosis such as translocation of phosphatidylserine across the plasma membrane and chromatin condensation together with a significant accumulation of HT29 cells at G2/M phase was detected at 48-72 h after treatment with 15 µM of SFN where the main signalling pathways affected were up-regulation of *Bax*, mitochondrial release of cytochrome c, and poly-(ADP-ribose)-polymerase (PARP) cleavage, without affecting p53 expression (Gamet-Payrastre et al. 2000). In contrast, Shen (2006) found that treating HT-29 cells with SFN concentrations ranging from 12.5 to 100 µM for 12 hours induced G1 cell cycle arrest dose dependently by down-regulating

cyclin D1, cyclin A, and c-Myc at both mRNA and protein levels and increasing the expression of p21 in a ROS- and MAP kinase-dependent, but p53-independent, manner (Shen et al. 2006). Similar results to those obtained by Gamet-Payrastre et al., (2000) have been observed in adenocarcinoma Caco-2 cells (Visanji et al. 2004), metastatic colon cells SW620 (Andelova et al. 2007; Rudolf et al. 2009), prostate cancer cells LNCaP (Chiao et al. 2002), and the human leukaemia HL60 cell line and its multidrug-resistant sublines (Jakubikova et al. 2005) using a wide variety of ITCs.

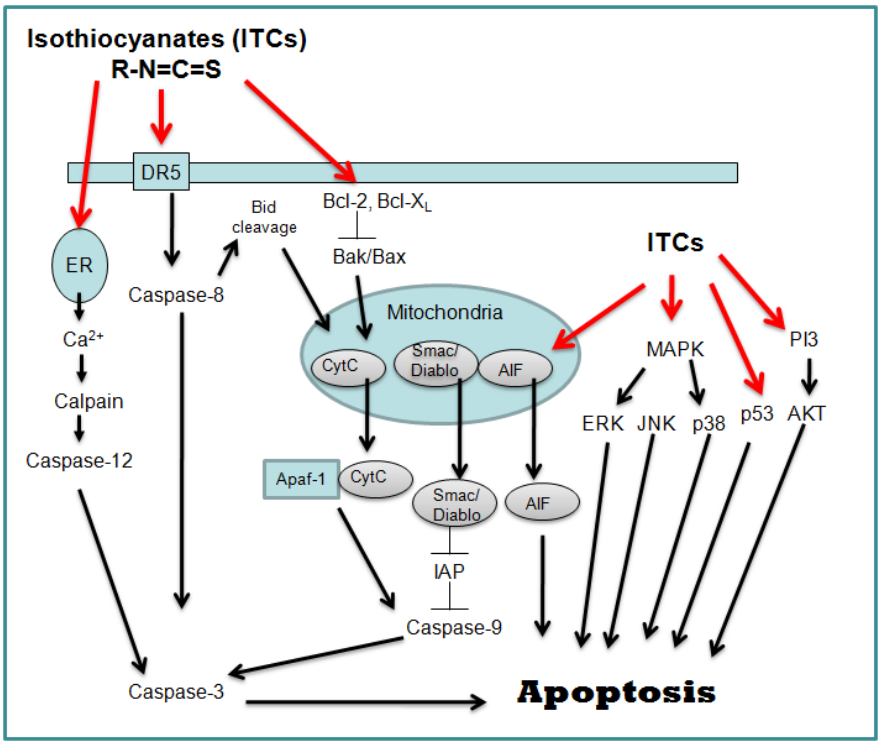


Figure 1.5 Mechanisms of isothiocyanate-induced apoptosis of tumour cells. Adapted from (IARC/WHO 2004)

Several independent mechanisms seemed to play important roles in this process including the activation of MAPK/c-Jun NH_2-terminal kinase (JNK), through which ITCs induce apoptosis. Activation of all three MAPKs (JNK, extracellular signal-regulated protein kinase (ERK) and p38 kinase) by phosphorylation (Figure 1.5) was observed in HT29 cells treated with phenethyl-ITC (PEITC). However, inhibition of JNK (but not ERK and p38)

suppressed apoptosis induced by PEITC (Hu et al. 2003). Another signalling pathway that has been found to participate in the induction of apoptosis by ITCs is PI3K/AKT. Pre-treatment of Caco-2 cells with a PI3K inhibitor (LY294002) was found to significantly decrease cell death induced by benzyl-isothiocyanates (BITC) (Jakubikova et al. 2005). Phenylmethyl isocyacyanate (PMITC) and PEITC were also shown to induce apoptosis in Jurkat and HL60 cells accompanied by an increase in the activity of JNK (Chen et al. 1998). Conversely, in Caco-2 cells exposed to AITC, BITC and PEITC expression of JNK 1/2/3 at the protein level was not affected and no phosphorylation of JNK was found; however the compounds induced a time and concentration dependent activation of ERK1/2 (Jakubikova et al. 2005). These conflicting results show significant variations among cells lines with respect to the potential apoptosis targets that are modulated by various ITCs and that more research is needed to clarify the mechanisms involved in how ITCs modulate a wide variety of cellular events such as proliferation, differentiation, growth arrest, and apoptosis.

1.4 Human Cell and Tissue Exposure to Isothiocyanates

An understanding of the hydrolysis of glucosinolates and absorption in human subjects would assist in the design of more robust molecular studies to evaluate the protective effects of *Brassica* against cancer and would guide the use of physiologically relevant ITC concentrations in human cell lines. Feeding trials with human subjects have shown that bioavailability of ITCs is greater following ingestion of raw Brassica with active plant myrosinase than after consumption of the cooked plant with denatured myrosinase (Conaway et al. 2000). Substantial amounts of glucosinolates occur in a wide variety of vegetables, and their occurrence has been extensively reviewed. For example, a minimum of ~12 mg of phenethyl isothiocyanate (PEITC) is released when 2 oz (56.8 g) of watercress is consumed (Hecht 1999). In a human study where volunteers were given a single serving of broccoli sprouts extract containing 200 μmol of ITCs of closely related chemical structures (composition: 77.2%

SFN/iberin and 22.8% erucin) (see Table 1.1) it was found that the ITCs were absorbed rapidly with peak plasma concentration of 0.94-2.27 µM 1h after ingestion (Ye et al. 2002). Likewise, Ji et al (2003) showed that the maximal plasma concentration (Cmax) of PEITC achieved 2.6 h post ingestion of 100 g watercress in humans (containing 25 mg of PEITC or 153 µmol if fully released), was 0.93 ± 0.25 µM (Ji and Morris 2003). In another study (Gasper et al. 2007) human subjects were fed with different broccoli soups (100 g of florets+150 ml water), one with standard broccoli containing 170 µmol of SFN and the other with high glucosinolate broccoli containing 573 µmol of SFN and it was found that the peak concentration of SF in the plasma was 2.2 and 7.3 µM, for standard and HG broccoli, respectively, and occurred 2 h after consumption of the soups.

Also, studies in laboratory animals have indicated that ITCs are promising chemopreventive agents against cancers at various sites. For instance, pre-treatment with SFN and or PEITC significantly decreased the total number of azoxymethane (AOM)-induced aberrant crypt foci (ACF) in Ficher rats (Chung et al. 2000). Similarly, ApcMin/+ mice fed with a SFN-supplemented diet developed significantly fewer and smaller polyps with higher apoptotic and lower proliferative indices in their small intestine, in a SFN dose-dependent manner equivalent to 5-10 µmol/day (Hu et al. 2006). In another *in vivo* study, benzyl isothiocyanates (BITC) reduced the incidence of mammary hyperplasia and carcinoma in a female mouse model with mammary-specific expression of the *neu* oncogene (Warin et al. 2009).

In conclusion, research conducted to date suggests that cruciferous vegetables contain constituents that reduce cancer risk. However, only limited information is available regarding the structure-activity relationship of different isothiocyanates. Therefore, this research examined if small differences in the structure of two related isothiocyanates, sulforaphane and iberin, in combination with two different selenium compounds can differentially regulate two specific selenoproteins, TrxR1 and GI-GPx, and if these differences

provide a guide to understanding their cancer preventive potential through modulation of oxidative stress in colon cancer cells.

1.5 Selenium and Cancer Prevention

Another dietary compound that has been shown to have cancer-chemopreventive roles is selenium (Se). Selenium is a mineral trace element that occurs in different chemical forms; it is toxic in large amounts, but essential at trace levels. The selenium content and species of both plant and animal foodstuffs depend on the quantity and chemical forms of the element to which they are exposed in the environment. These factors, affecting selenium bioavailability, have recently been the focus of debate and research to derive better data on selenium requirements and reliable biomarkers of selenium status to ultimately establish dietary recommendations for optimal health (Fairweather-Tait et al. 2010; Hurst et al. 2010).

Although dietary selenium intake was shown to have inverse associations with cancer mortality as early as the 1960s (Shamberger and Frost 1969), it was not until 1996 that an intensive effort was launched to try to understand the mechanism of action of Se as a cancer preventive agent. Clark and co-workers reported results from The Nutritional Prevention of Cancer (NPC) study, where they showed that supplementation with selenized yeast decreased cancer incidence by nearly 50% (Clark et al. 1996). After this report extensive epidemiological evidence has emerged showing that dietary selenium deficiency is linked to an increased risk of cancers in several organs including lung (Zhuo et al. 2004), oesophagus (Wei et al. 2004), stomach (Corella et al. 1996), prostate (Li et al. 2004) and colon (Jacobs et al. 2004). However, results are heterogeneous among organs (Rayman 2005). For instance, despite the fact that studies investigating an association between selenium and cancer in the gastrointestinal tract have produced promising results to support a chemopreventive role of selenium, several others have produced inconclusive evidence, and as a consequence the World Cancer Research Foundation has

concluded in its latest report that there is limited evidence that food containing selenium protects against colorectal and stomach cancer, but for other sites, such as prostate cancer, such foods probably decrease risk (WCRF/AICR 2007). In a recent systematic review aimed to study the beneficial and harmful effects of antioxidant supplements such as beta-carotene, vitamin A, vitamin C, vitamin E, and selenium in preventing gastrointestinal cancers the authors concluded that these supplements, with the possible exception of selenium, might increase mortality (Bjelakovic et al. 2008). A disappointing outcome arose from the recent Selenium and Vitamin E Cancer Prevention Trial (SELECT), which demonstrated that selenium (200 µg/d from *L*-selenomethionine), vitamin E, or selenium + vitamin E (at the tested doses and formulations) did not prevent prostate cancer in the generally healthy, heterogeneous population of men in the study and also had no effect on secondary endpoints, which included lung cancer and colorectal cancer (Lippman et al. 2009). In addition, these supplementation trials have shown recently that a high level of serum selenium correlates with increased metabolic risk factors including type II diabetes, blood pressure and lipid levels (Stranges et al. 2007; Stranges et al. 2009).

Potential limitations of SELECT include that selenized yeast, as employed by Clark et al., was not tested in this trial. It is feasible that this difference proved important and that other selenium species observed in the 'Clark' yeast (Uden et al. 2004; Amoako et al. 2009) were connected to those previous results. In fact, in addition to selenomethionine (initially thought to be the major Se form, but found later to represent no more than 20%) other compounds identified later included selenocysteine, Se-methylselenocysteine and selenomethionine (representing ~20%). Thus the selenized yeast actually contained a cocktail of selenium in a variety of chemicals forms (Ip 1998). Also, different doses of selenium were not tested in the SELECT trial.

In spite of inconsistencies, a large body of experimental evidence in animals and cell culture models indicates that this essential trace element exerts

anticarcinogenic effects by multiple mechanisms including altered carcinogen metabolism, cell cycle regulation, immune surveillance, cell death programming, cancer cell migration and angiogenesis (Jackson and Combs Jr 2008; Zeng and Combs Jr 2008). Some such functions may relate to the crucial fact that selenium is a component of the amino acids selenocysteine and selenomethionine, which are incorporated into 25 genes encoding over 30 mammalian selenoproteins, as some genes (such as *GPx4*) have alternative splice variants (Kryukov et al. 2003). Selenoproteins that might be relevant to cancer risk include GPx1, GI-GPx, Sep15, SelP and TrxR1 (Bellinger et al. 2009) Single nucleotide polymorphisms (SNPs) or mutation in some of these genes have been related to cancer. For instance, loss of heterozygosity of cytosolic glutathione peroxidise (GPx1) was identified in 42% of cases of colon cancer and was also a feature of others cancers including breast, prostate, lung, head and neck cancer (Hu et al. 2005). Other polymorphisms in GI-GPx, and in SelP, have been associated with colorectal cancer, although these SNPs seem to play a minor role in colorectal carcinogenesis (Al-Taie et al. 2004), whereas a particular Sep15 SNPs may increase lung cancer risk in smokers (Jablonska et al. 2008).

Efficacy of high-level selenium supplementation in cancer prevention studies has been proposed to be due to the direct chemical properties of selenium and its metabolites rather than being mediated through selenoproteins (Chu et al. 2004) as will be point out in section 1.5.2. Functions of specific selenoproteins during the carcinogenesis process and the interaction of selenium with other food compounds, such as isothiocyanates, in the modulation of selenoprotein expression awaits further investigation.

1.5.1 Selenoprotein Biosynthesis

The biosynthesis and specific incorporation of selenocysteine (Sec) into selenoproteins requires a unique mechanism specified by the UGA codon in mRNA. Unlike the other 20 amino acids, biosynthesis of Sec takes place on a

unique tRNA species ($tRNA^{Sec}$) from selenophosphate as the Se source. However, as UGA is also a stop codon this process requires multiple features such as the presence of a stem-loop structure in the 3'-mRNA-untranslated region, termed the selenocysteine insertion sequence element (SECIS), which recruits several protein factors such as the SECIS-binding protein (SBP2) and the selenocysteine elongation factor (EFSec) (Figure 1.6) (Hatfield et al. 2006; Papp et al. 2007).

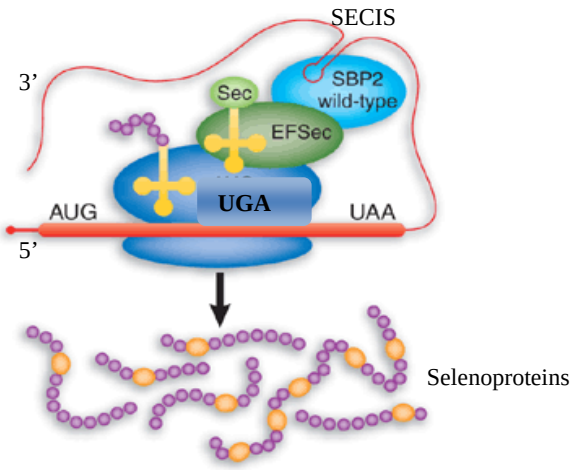


Figure 1.6 Selenoprotein synthesis. The SECIS element in the 3' untranslated region of the mRNA (stem loop in red) recruits SBP2 (light blue), which in turn recruits EFSec (green) and $tRNA^{Sec}$ (yellow). The complex interacts at the ribosome to decode UGA as selenocysteine. Adapted from (Berry 2005).

1.5.2 Metabolism of Different Dietary Selenium Compounds

Most dietary forms of selenium are easily absorbed, but subsequent metabolism depends on the form in which they are present. In general selenomethionine, selenocysteine, selenate and selenite enter the selenide pool to be subsequently used for selenoprotein synthesis or excreted in the urine as a selenosugar. The major forms of selenium occurring in food are the organic forms selenomethionine (SeMet, found in plants and animal sources) and selenocysteine (found in animal sources). Selenocysteine is cleaved by a β-lyase directly into selenide, whereas SeMet has first to be "trans-sulfurated"

into selenocysteine or, alternatively, SeMet can be incorporated non-specifically into proteins (Brigelius-Flohé and Banning 2009). Inorganic salts such as selenate and selenite are provided mostly by drinking water and are also contained in some foods in low quantities (Fairweather-Tait et al. 2010)

There is extensive evidence that monomethylated forms of selenium are critical metabolites for the chemopreventive effect of selenium (Ganther 1999). Indeed, Clement Ip and co-workers have produced strong experimental evidence that any precursor that will directly generate a steady stream of methylselenol (CH_3SeH) or its derivate, is more active than selenite or selenomethionine in tumour inhibition (Ip et al. 1991; Ip et al. 2000). As mentioned above, selenium accumulating plants produces predominantly selenomethionine, which is metabolised via the multi-step transsulfuration pathway to selenocysteine, in turn degraded to hydrogen selenide (H_2Se) for subsequent methylation by methyltransferases that give rise to methylselenol (Ip et al. 2002). In this respect, Se-methylselenocysteine (SeMSC) (present in plants of the Brassica family) and γ-Glutamyl-Se-methylseloncysteine (present in plants of the Allium family), which are known to be converted to methylselenol (Dong et al. 2001), have been found to account for the anticarcinogenic effect of selenium-enriched broccoli and garlic respectively and have proved to be more effective in reducing colon and mammary tumorigenesis in rodents than selenate or selenium-enriched yeast, which contains mostly selenomethionine (Finley et al. 2000; Ip et al. 2000) (Figure 1.7). Finally, these studies have made evident that the degree of methylation is an important factor affecting the anticarcinogenic activity of selenium and in this respect selenomethionine is not as efficient as SeMSC in generating methylselenol. Therefore, the chemoprotective properties of these enriched-vegetables may be a result of the form of selenium present in them that allows more selenium to enter the cancer-protective pool.

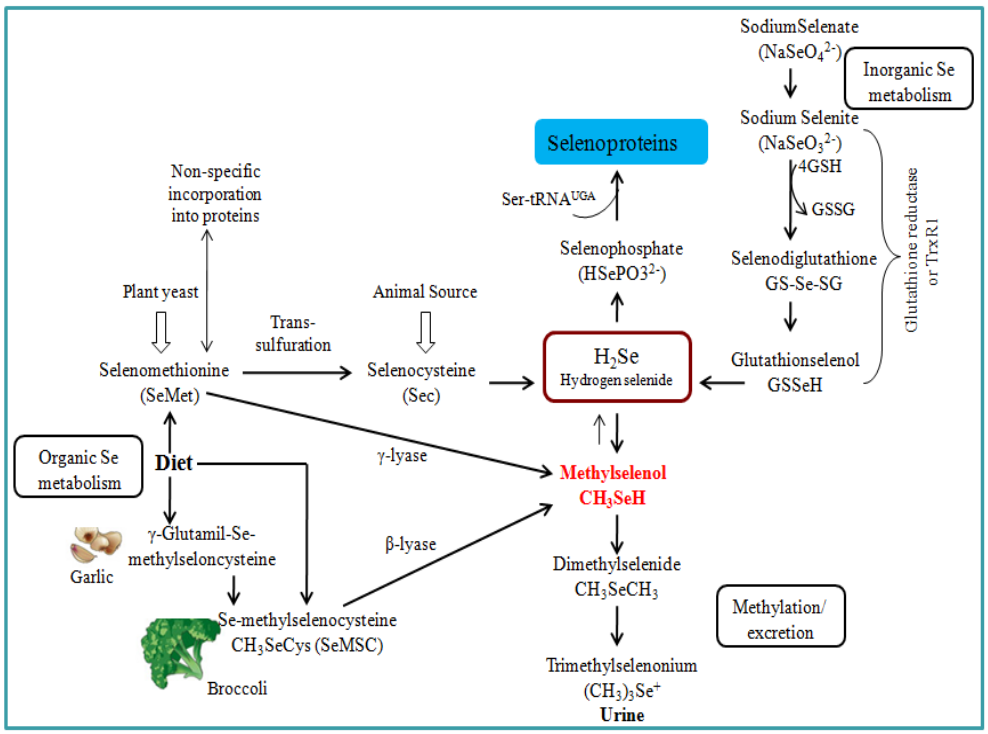


Figure 1.7 Pathways of selenium metabolism. Selenium salts undergo reductive metabolism with glutathione reductase and NADPH or can be directed reduced by thioredoxin reductase (*TrxR1*). Selenomethionine and selenocysteine can generate methylselenol through hydrogen selenide. The latter is the source for incorporation into Sec-containing proteins by co-translational incorporation into Ser-tRNA^{UGA}. Diet may also enrich the methylselenol pool bypassing the hydrogen selenide pool, by using direct precursors such as Se-methylselenocysteine or γ-Glutamil-Se-methylseloncysteine, which is converted to SeMSC first and then acted upon by a β-lyase to give also methylselenol. Methylation of hydrogen selenide constitutes an excretion pathway for selenium (Combs and Lu 2006; Brigelius-Flohé and Banning 2009).

1.5.3 Antioxidant Effects of Selenium

Free radicals are known to play an important role in many diseases, including cancer. Since free radicals and reactive oxygen species are continuously produced in vivo, mammalian organisms have evolved to possess not only antioxidant and electrophile defense systems to protect against them, but also repair systems that prevent the accumulation of oxidatively damaged molecules (Hanausek et al. 2004). Because of the highly cytotoxic and reactive nature of reactive oxygen and nitrogen species, their accumulation must be under tight control and in this regard selenoproteins has been shown to reduce oxidative

stress and limit DNA damage, both of which have been associated with cancer risk.

Well-characterised selenoproteins are the families of glutathione peroxidases (GPxs), thioredoxin reductases (TrxR) and iodothyronine deiodinases (Beckett and Arthur 2005). More recently selenoprotein P has become a focus of research, as it has been considered a good index of human selenium nutritional status (Burk and Hill 2005), but has also been implicated in tumorigenicity and metastasis of colon cancer cells (Irons et al. 2010). However, this section will be used to review briefly the function of the first two families, which are involved in cell antioxidant systems.

1.5.3.1 Glutathione Peroxidases

There are six known GPxs which contain selenocysteine at the active site. Glutathione peroxidise 1 (GPx1), or cytosolic glutathione peroxidase, is a highly efficient antioxidant enzyme that catalyzes glutathione-dependent hydroperoxide reduction and is also found in the mitochondria. GI-GPx or GPx2, also known as gastrointestinal-GPx, has an unusual distribution. In rodents, it is exclusively expressed in the gastrointestinal tract (in humans also in liver), which suggests a specific function as a primary barrier against the absorption of dietary lipid hydroperoxides (Wingler and Brigelius-Flohe 1999). This gene has been also found to be expressed in human breast cancer cells (Chu et al. 1999). GPx3 is an extracellular GPx from human plasma that can reduce H_2O_2, fatty acids hydroperoxides, and phospholipid hydroperoxides, but not cholesterol hydroperoxides (Yamamoto and Takahashi 1993). GPx4, present in cytosol and mitochondria, reduces phospholipids, cholesterol, and thymine hydroperoxides (Bao et al. 1997). GPx5 and GPx6 are readily detected in mouse epididymis and olfactory epithelium, respectively (Kryukov et al. 2003).

Even though ROS are clearly involved in inflammation and selenium has cancer protective effects in a variety of experimental system the role of selenoproteins in these pathologies was uncertain until mice with disrupted *GPx1* and *GPx2* genes, *GPx1*/2-KO, were proven to develop ileocolitis at a young age as a result of losing virtually all GSH-dependent activity to reduce H_2O_2 (Esworthy et al. 2001). In these animals the lack of *GI-GPx* was more detrimental, since one intact *GI-GPx* allele was sufficient to prevent intestinal inflammation (Esworthy et al. 2005). Similarly, the same group developed an ileal and colonic cancer model using bacteria-induced mice in which *GPx1* and *GI-GPx* were disrupted (Chu et al. 2004). Furthermore, in transgenic mice carrying a mutant selenocysteine transfer RNA gene, which causes reduced selenoprotein synthesis, an increased number of preneoplastic lesions for colon cancer was observed when compared with wild type mice (Irons et al. 2006). Surprisingly, in a study carried out to examine the preventive effect of selenoproteins and the selenocompound selenite in transgenic mice with co-expression of *TGFα*/*c-Myc*, which generates an oxidative environment prior to hepatocarcinogenesis development, it was found that selenoprotein deficiency resulting from a Se-deficient diet containing 0 ppm significantly suppressed hepatic tumour formation, whereas a clear correlation between increased selenoprotein expression and tumour formation was seen in mice fed with 0.4 ppm Se (Novoselov et al. 2005), findings consistent with the known inhibitory effect of antioxidant selenoproteins on apoptosis (Fu et al. 2001).

These data highlight the complexity of selenoprotein regulation and the need for more studies to understand the biochemical basis for the protective effect of selenoproteins against cancer.

1.5.3.2 Thioredoxin Reductases

The thioredoxin reductases, with thioredoxin (Trx) as a substrate and NADPH as a cofactor, form a powerful dithiol-disulphide oxido-reductase system that regulates the cellular redox state of cells (Beckett and Arthur 2005). This

system in conjunction with GSH are the two main antioxidant systems that reduce thiol (-SH) groups. Three mammalian TrxR selenoenzymes have been identified: the cytosolic enzyme TrxR1, the mitochondrial enzyme TrxR2, and a testis-specific enzyme thioredoxin-glutathione reductase (TGR/TRxR3), the latter also possessing glutathione and glutaredoxin reductase activity

The Trx system provides electrons to ribonucleotide reductase, which is essential for DNA synthesis by converting ribonucleotides to deoxyribonucleotides, as well as promoting the catalysis of thioredoxin peroxidases (peroxiredoxins)—important enzymes in the defence against oxidative stress and protein disulfide-isomerase (PDI), which catalyzes protein disulfide formation within the endoplasmic reticulum (ER). Additional substrates include two ER proteins with Trx-domains, calcium-binding protein 1 and 2 (CaBP1 and CaBP2) involved in calcium metabolism (Papp et al. 2007). The thioredoxin system also plays a central role in the regulation of gene expression via redox control of transcription factors including NF-κB (Matthews et al. 1992), the AP-1/Ref1-dependant pathway (Hirota et al. 1997), P53 (Ueno et al. 1999), glucocorticoid receptor, and apoptosis-regulating kinase (ASK1), thus indirectly regulating cellular activities such as cell proliferation, cell death, and immune-response activation (Saitoh et al. 1998; Arnér 2009) (Figure 1.8). In addition, a group of selenium compounds including selenite, selenodiglutathione, methylseleninate, selenocystine, and ebselen are substrates of TrxR (Ganther 1999; Zhao and Holmgren 2002). Among these compounds selenodiglutathione and selenite are metabolized to hydrogen selenide, the selenium donor for Sec biosynthesis (Figure 1.7).

Recently, elevated expression of the thioredoxin system has been implicated in increased proliferation of tumour cells, low apoptosis rate, aggressive tumour growth, and decreased patient survival (Lincoln et al. 2003; Raffel et al. 2003). However, even though Trx and TrxR1 have been found to be over-expressed in many malignant cells, this system is very important in the area of redox regulation and can bring about direct antioxidant support such as the removal

of hydrogen peroxide species during the initial stages of the disease, where the thioredoxin system can protect the cells against the oxidative stress associated with cancer. However, during the later stages of the disease, the growth-promoting anti-apoptotic property of the thioredoxin system on cancerous cells outweighs its beneficial antioxidant properties (Maulik and Das 2008), as shown by Raffael and co-workers in colon cancer patients where increased thioredoxin-1 expression was a relatively late event in colorectal carcinogenesis (Raffel et al. 2003).

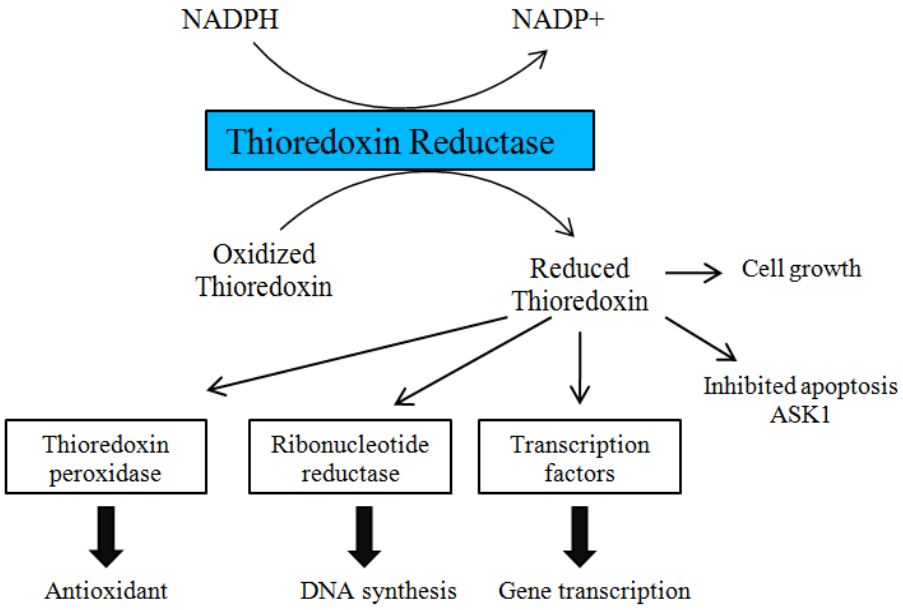


Figure 1.8 Functions of the thioredoxin system

In summary, it is important to continue investigating the cellular functions of important selenoproteins such as GI-GPx and TrxR1 involved in the maintenance of redox status and signal transduction in colon cancer cell to shed light on functions of selenium in cancer development.

1.6 Colorectal Carcinogenesis

Worldwide, cancer is still one of the major public health problems and among them colorectal cancer is the third most common malignancy in the developed world after lung and breast cancer (Acheson and Scholefield 2008). Wide geographical variation in cancer incidence and mortality rates is thought to be due to lifestyle and environmental factors, including diet (Martinez et al. 2008). However, despite all the research input in the past 20 years aimed to study the links between diet and cancer, it is still uncertain whether fruit and vegetables protect against colorectal cancers (Nomura et al. 2008).

The evolution of colorectal cancer is a multi-step process whereby progression stages range from normal epithelium to aberrant crypt foci (ACF) (Roncucci et al. 2000), to the development of benign adenomatous polyps, and finally to invasive cancer and metastasis (Fearon and Vogelstein 1990). In the gastrointestinal tract, intestinal metaplasia precedes the development of carcinoma in the stomach and in Barrett's oesophagus, and in the colon; the adenoma is now universally regarded as the immediate antecedent of most colorectal carcinomas (Figure 1.9). However, it is becoming increasingly clear that colorectal cancer is a more heterogeneous disease than had previously been recognized as normal-appearing rectal mucosa from participants with adenoma or adenocarcinoma has been reported to have field defects (Alberts et al. 2007). This has been evidenced by proteomic, transcriptomic and more recently epigenomic analysis (Belshaw et al. 2008; Bernstein et al. 2008).

The initial genetic change in most colorectal adenomas is thought to be somatic mutations in the tumour suppressor gene adenomatous polyposis coli (*APC*) as this gene is responsible for binding and down-regulating β-catenin. When *APC* is inactivated it fails to regulate apoptosis, cell cycle progression and chromosomal stability, allowing β-catenin to translocate from the lateral cell membrane to the nucleus where it promotes transcription of multiple genes implicated in tumour growth and invasion through the activation of the Wnt

cascade (Humphries and Wright 2008), possibly one of the most important pathways for the control of intestinal epithelial stem cell function (McDonald et al. 2006).

However, studies have indicated that *APC* mutation is not obligatory in all cases of colorectal cancer, even as a late event. In addition to this classical pathway at least two other different pathways to colorectal cancer have been proposed. The first involved the inactivation of genes required to repair base-base mismatches in DNA, generally referred to as mismatch-repair (MMR) genes, primarily *MLH1* and *MSH2*. This inactivation can be inherited, as in hereditary nonpolyposis colon cancer (HNPCC), or acquired through methylation-associated silencing. In patients with HNPCC, germ-line defects in MMR genes confer a lifetime risk of colorectal cancer of about 80%. Somatic inactivation of MMR genes occurs in about 15% of patients with nonfamilial colorectal cancer. In these patients, biallelic silencing of the promoter region of the MMR genes by promoter methylation inactivates MMR (Markowitz and Bertagnolli 2009). These errors, which generate deletions and insertions of some nucleotides more frequently at the level of repeated sequences, are named microsatellites instability (MSI), in which the inability to repair strand slippage within repetitive DNA sequence elements changes the size of the nucleotide repeats (Kruhoffer et al. 2005).The second pathway involves the formation of a hamartoma as a precursor lesion, in which the mutated bone morphogenetic protein (BMP) signalling pathway has been identified to predispose to colorectal cancer (Hardwick et al. 2008). Recent data also show that suppression of *CDK8* gene expression inhibits proliferation of colon cancer cells, alleviating β-catenin hyperactivity in the canonical WNT/β-catenin pathway that contributes to growth, invasion and survival of cancer cells (Firestein et al. 2008).

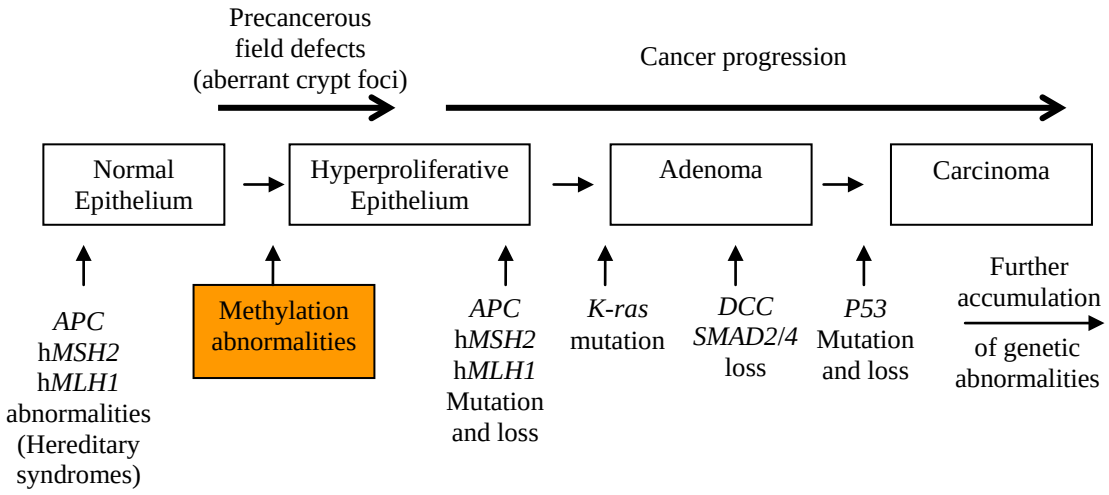


Figure 1.9 An adaptation of the Vogelstein adenoma-carcinoma sequence**.** Sequence of mutational events that involve the transition from normal colon epithelium to premalignant adenoma and then invasive adenocarcinoma. Loss of function of the adenomatous polyposis coli (*APC*) gene, which encodes a protein involved in cell adhesion and transcription, is found in up to 85% of all cases of colorectal cancer (CRC). Mutations in the mismatch-repair genes *MLH1* and *MSH2* contribute to genetic instability. The oncogene K-ras, a GTP-ase that controls cell proliferation, is mutated in 50–60% of cases of CRC (Ray 2010). The deleted in colorectal cancer gene (*DCC*) might be involved in normal cell-cell and/or cell extracellular matrix interactions. SMAD2/4 is involved in the transforming growth factor-β signaling pathway to suppress epithelial-cell growth and *P53* mutations tend to be late events and increase the resistance of cancer cells to apoptosis. Methylation events are found in early cancer stages. Adapted from (Geenen et al. 2003; Kerr 2003).

Among the factors affecting mucosal integrity it has been long recognised that both exogenous carcinogens and endogenous biological processes are known to cause mutations, affecting genes involved in the maintenance of DNA stability (Greenblatt et al. 1994). Considering the multiplicity of proteins involved in DNA synthesis, DNA repair, mitosis, and the cell cycle, the number of potential gene targets that could interfere with replication fidelity in somatic cells is large. Abnormalities in some of these processes could result in the mutator phenotype (Loeb 2001), which could increase the probabilities of both neoplastic transformation and the generation of increasingly malignant subclones during tumour progression. For decades the mutational theory of cancer was accepted as the main contributor of neoplasia (Nowell 1976; Edler and Kopp-Schneider 2005). However, a large amount of data indicating the importance of epigenetic processes, especially those resulting in the silencing

of key regulatory genes, has led to the realization that genetics and epigenetics cooperate at all stages of cancer development (Jones and Baylin 2007).

1.6.1 Caco-2 Cell Line as an in vitro Model to Study Colorectal Cancer

Animal models and human studies have been used widely to demonstrate underlying mechanisms that link specific dietary constituents to cancer risk. However, the simultaneous fluctuations of fuels, hormones, genotypes and the complexity of tissue and organ organization in experimental *in vivo* models make it very difficult sometimes to demonstrate molecular mechanisms of specific regulation. In addition, *in vitro* models are costly and not suitable for initial antioxidant screening of food compounds (Liu and Finley 2005; Goya et al. 2009). Nevertheless, in analysis of data from *in vitro* experiments one should consider a number of limitations, as cultured cells will not be fully representative of the tissue from which they were derived due to the different cell microenvironment that characterise the *in vivo* condition. Providing that the limitations of the models are appreciated, cell culture is a valuable, if not the most valuable, tool in biomedical science (O'Brien et al. 2000).

In cancer biology, cell lines are often used instead of primary tumours because of their widespread availability and close reflection of the *in vivo* state (Kleivi et al. 2004). The Caco-2 cell line was established from a moderately well-differentiated colon adenocarcinoma obtained from a 72-year-old patient (Fogh and Orfeo 1977). Undifferentiated Caco-2 cells are characterized by a proliferative state where cell cycle, signal transduction, DNA metabolism, transcription, nucleocytoplasmic transport and protein biosynthesis are predominantly altered by the underlying pathology (Bédrine-Ferran et al. 2004). Although this is a colonic tumour derived cell line, it differentiates post-confluence. After this period Caco-2 cells start acquiring an enterocyte-like phenotype and develop the capacity to detoxify drugs and xenobiotics, to protect the monolayer, and to efficiently transport water and ions, in addition to

lipids and amino acids (Bédrine-Ferran et al. 2004; Laitinen 2006). However, these studies will be carried out in the undifferentiated cancer colonic phenotype of Caco-2 cell rather than in the enterocytic-type to study specific antioxidant and epigenetic chemopreventive effects of selenium and isothiocyanates.

1.7 Epigenetics

Epigenetics refers to heritable changes in the pattern of gene expression without any alteration in the nucleotide sequence (Esteller 2005). Epigenetic mechanisms are involved in embryonic development and the establishment of tissue-specific expression, X-chromosome inactivation and imprinting patterns, and maintenance of chromosome stability (Lopez-Serra and Esteller 2008). The factors known to be involved in epigenetic regulation of chromatin structure and gene activity are DNA methylation, histone modifications, chromatin remodelling factors and non-coding regulatory RNAs (Ross et al. 2008) and among these DNA methylation is one of the most important mechanisms for epigenetic silencing of gene expression in mammals.

1.7.1 DNA Methylation

Different forms of methylation occur in different organisms. In humans and other mammals the most frequent epigenetic modification of DNA bases involves cytosine, which is modified reversibly by adding a methyl group (CH_3) to its carbon 5-position. In this reaction DNA methyltransferases (DNMTs) catalyze the transfer of the methyl group from S-adenosylmethionine (SAM) to the 5 position of cytosine in DNA, producing 5-methylcytosine and S-adenosylhomocysteine (SAH) (Figure 1.10).

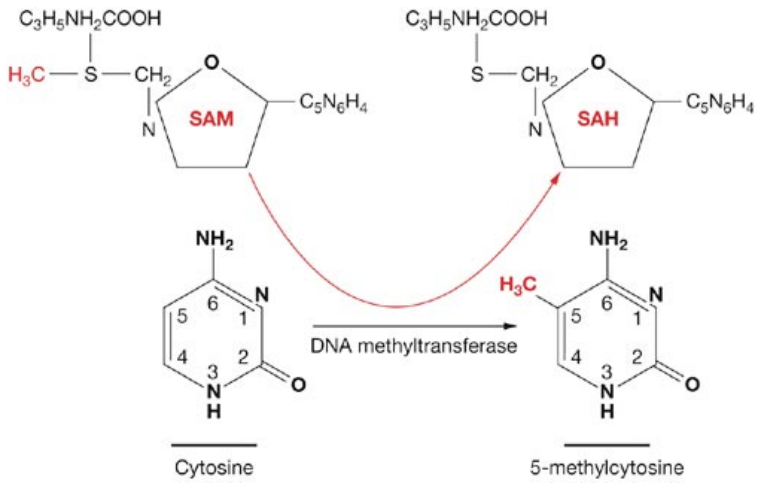


Figure 1.10 Biochemistry of DNA methylation. Adapted from (Richardson 2007)

In humans and other mammals this modification occurs only on cytosines that precede a guanosine in the DNA sequence, referred as the CpG dinucleotide (Herman and Baylin 2003). This dinucleotide is under-represented in much of the genome because it is prone to methylation and spontaneous deamination, causing conversion from 5-methylcytosine to thymine, which is subject to ineffective DNA repair. However, short regions of 0.5-4Kb in length, known as CpG islands, are rich in CpG content. These islands are typically found in or near promoter regions of genes where transcription is initiated. In contrast to the vast amount of genomic DNA, in which most CpG islands are heavily methylated (major fraction), CpG islands in promoters of normal somatic cells remain unmethylated (minor fraction), allowing gene expression to occur (Baylin 2005) (Figure 1.11). In most cases, gene expression patterns need to be maintained during cell division and passed to the daughter cells. For this purpose, epigenetic memory mechanisms, which are carefully controlled by DNA methyltransferase enzymes, have evolved (Van Steensel 2005).

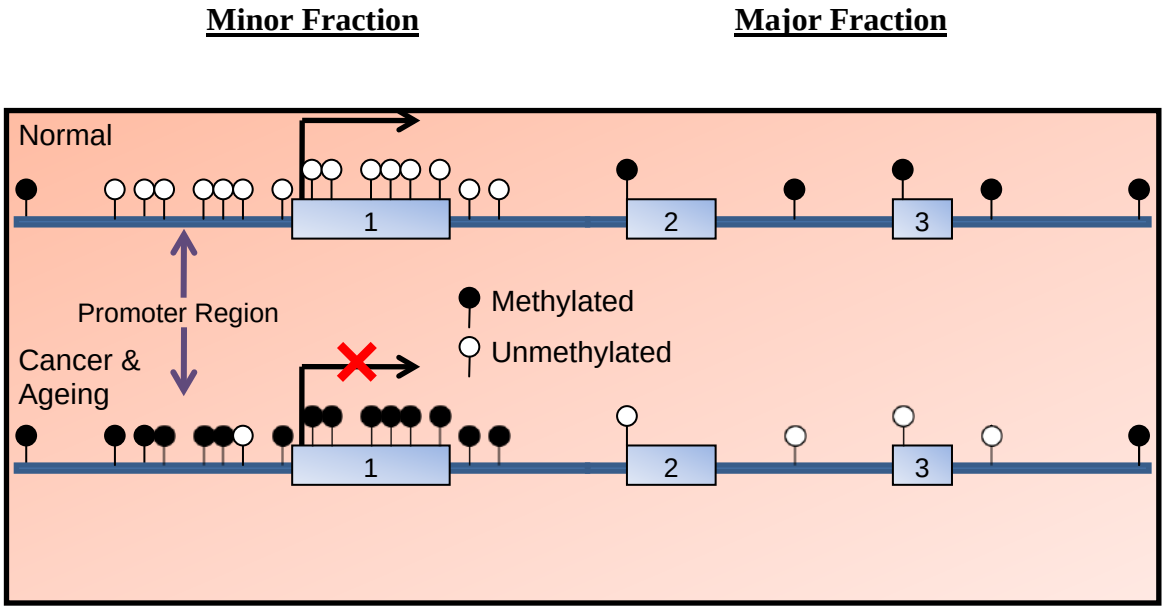


Figure 1.11 Distribution of CpG islands in the human genome. The methylated state in the major fraction in normal cells may help suppress unwanted transcription, while the unmethylated state of the CpG islands in the 5' end of the gene allows active gene transcription (arrow in upper panel). In cancer cells, this pattern of CpG methylation becomes disrupted with high level of methylation at the promoter region causing abnormal gene silencing. Adapted from (Herman and Baylin 2003)

1.7.1.1 DNA Methyltransferases

Patterns of DNA methylation are disturbed in important human diseases such as colon cancer and an understanding of how these marks are set up and maintained is of great significance. There are three known biologically active *DNMT*s in mammalian cells with different roles: DNMT1, DNMT3A and DNMT3B. DNMT1, with preferential activity for hemimethylated DNA, acts mainly as maintenance methyltransferase and DNMT3A and DNMT3B are mainly involved in *de novo* methylation (Herman and Baylin 2003). It is generally thought that patterns are established during embryonic development (DNMT3A and DNMT3B) and are then faithfully inherited in somatic cells by a 'maintenance' mechanism (DNMT1). However, an updated model for DNA methylation maintenance has been proposed in which all three DNMTs not

only cooperate but also may possess both *de novo* and maintenance functions *in vivo* (Jones and Liang 2009). In addition, it is well known that DNA methylation can be removed passively by blocking methylation of newly synthesized DNA during DNA replication, but recent data also suggest that active DNA demethylation is initiated by the same enzymes that establish the methylation mark in first place, the DNMT3A and DNMT3B (Ooi and Bestor 2008), and perhaps by a base excision repair pathway (Zhu 2009).

1.7.1.2 Repression of Tumour Suppressor Genes

Hypermethylation of the CpG islands in the promoter regions of tumour-suppressor genes (commonly unmethylated) is a major event in the origin of many cancers and is correlated with the loss of active gene expression (Figure 1.11). The large number of genes found to undergo hypermethylation in various malignancies has implicated a role for epigenetic changes in the initiation and/or progression of cancer affecting many of the cellular pathways involved in this process (Table 1.3) (Herman and Baylin 2003; Baylin 2005). A gene hypermethylation profile carried out by Esteller and co-workers, to unfold the critical aspects related to methylation and cancer, provided an opportunity to examine the pattern of inactivation of such genes among different tumours (Esteller et al. 2001), suggesting that the profile of hypermethylation of the CpG island in tumour-suppressor genes is specific to the cancer type. For instance, gastrointestinal tumours (colon and gastric) have been shown to share a set of genes undergoing hypermethylation characterized by $p16^{INK4a}$ (Abbaszadegan et al. 2008), $p14^{ARF}$ (Esteller et al. 2000), *MGMT* (Hee et al. 2003), *APC* (Esteller et al. 2000) and h*MLH1* (Kane et al. 1997). In addition to these genes, there is growing interest in the study of hyperplastic polyposis protein 1 (*HPP1*) as this gene has been found to be silenced in a number of tumour types, suggesting a potential role as a tumour suppressor. Methylation of this gene is detectable not only in the serum of patients with colorectal cancer but also in patients with pre-malignant adenomas and has been suggested as a biomarker in gastrointestinal malignancies (Elahi et al. 2008).

Among the loci that can undergo aberrant methylation in colorectal cancer (CRC), a subgroup seems to become aberrantly methylated as a group and is referred to as the CpG island methylator phenotype (CIMP) (Toyota et al. 1999). In an attempt to establish CIMP as a distinct marker for CRC Laird and colleagues analysed the methylation of 195 individual CpG islands in 295 CRCs and from their results, they proposed a panel of genes to standardize the classification of CIMP CRC (Weisenberger et al. 2006). Lack of agreement has generated several reports of CIMP subgroups according to the frequency of CpG island methylation in CRC (Shen et al. 2007; Nagasaka et al. 2008; Ang et al. 2010)

Table 1.3 Pathways Disrupted by Gene-Promoter Hypermethylation and Associated Gene Silencing in Cancer.

Pathway	Genes
Altered cell cycle control	*p16, p15, p14, p73*
Repair of DNA damage	*MLH1, MGMT, GST-Pi, BRCA1*
Apoptosis	DAP Kinase, caspase 8
Tumour-cell invasion or tumour architecture	E-cadherin, *VHL*, *APC*
Growth-Factor response	*ESR1*, *RAR-β*

Other abnormalities in cytosine methylation have been attributed to physiologic ageing, which is associated with *de novo* CpG island methylation in human colorectal mucosa and has been suggested as one of the earliest mechanisms predisposing to tumorigenic transformation. For example, the CpG island associated with the human estrogen receptor (*ESR1*) has been observed to be hypermethylated with ageing in the colonic mucosa (Issa et al. 1994; Belshaw et al. 2005), altering growth regulation of colonic cells and supporting the concept that age-dependent changes in DNA methylation might contribute to the global and promoter-specific epigenetic changes in colon.

In this regard, studies have shown that global DNA methylation content is decreased during colonic carcinogenesis (Shuji et al. 2008) and although aberrant methylation changes such as global hypomethylation and regional promoter hypermethylation are two processes that occur simultaneously in cancer cells no relationship among them has been found, suggesting that the contribution of DNA hypomethylation to tumorigenesis is separate from that of transcriptional silencing induced by CpG island hypermethylation in colorectal neoplasia (Bariol et al. 2003).

Importantly, these aberrant methylation changes have been found to be modulated by a diverse range of nutrients, supporting a role of bioactive food compounds on the human genome to alter the expression of genes and gene products (Trujillo et al. 2006). It is now well known that essential and nonessential food components, such as Se and ITCs, alter a number of cellular mechanisms associated with health and disease prevention, including carcinogen metabolism, cell signalling, cell cycle control, angiogenesis and apoptosis (Keck and Finley 2004; Bertl et al. 2006). Interestingly, these are also processes that are likely regulated by epigenetic events to impact gene function and chromatin stability. Thus, research identifying links between nutrition and cancer progression could address the importance of bioactive food components such as ITCs and other essential micronutrients such as Se to regulate epigenetic events for cancer prevention.

1.8 Diet and Epigenetics

The most widely investigated nutrients to establish causal links between diet and the epigenetic role of DNA methylation in cancer are those nutrients involved in one carbon metabolism (Figure 1.12) because of their involvement in the synthesis of SAM. Early experimental studies found that dietary methyl group deficiency leads to liver carcinogenesis in rodents in the absence of any additional carcinogenic treatment (Mikol et al. 1983). Similarly, a methyl group–deficient diet increased the incidence of colonic neoplasia in dimethylhydrazine-treated rats (Cravo et al. 1992). These data were also corroborated by epidemiological studies that found an inverse relationship between dietary folate and the incidence of colorectal adenomas in humans (Giovannucci et al. 1993). Other studies have, however, found no association between cancer risk and a combined low folate and high alcohol intake (Flood et al. 2002; Harnack et al. 2002). Folate supplementation has been found to increase genomic DNA methylation in the liver of ageing rats (Choi et al. 2005) and to accelerate age-dependent methylation of the *ESR1* CpG island in mouse colon (Belshaw et al. 2005). In this respect, the effects of folate status on DNA methylation and potentially on colon carcinogenesis are still controversial as animal studies report a reduction in early markers of colon cancer, such as aberrant crypt foci (ACF), when folic acid is administered prior to development of cancer (Kim et al. 1996). However, tumorigenesis is accelerated if folic acid is given after the lesion has emerged, perhaps through provision of DNA precursors for cancer cell growth (Song et al. 2000).

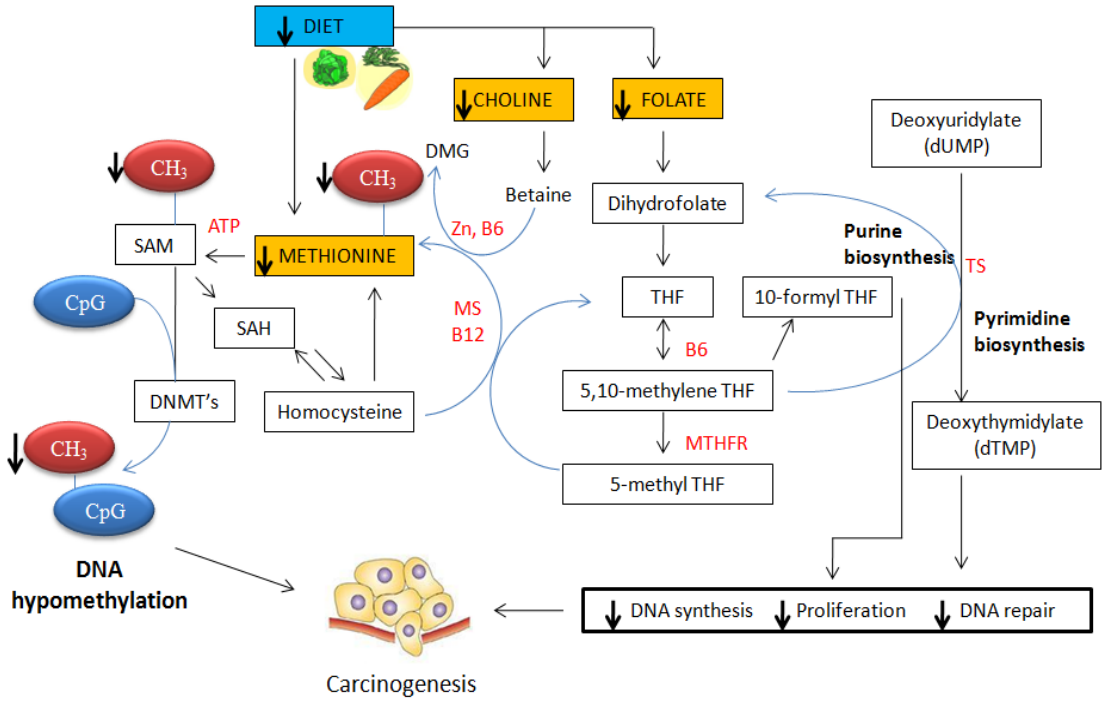


Figure 1.12 Folate and one-carbon metabolism: regulation of DNA synthesis, repair and methylation. A simplified scheme showing how nutrients involved in one carbon metabolism (yellow box) mediate normal DNA synthesis, repair and methylation and how its deficiency might impact on colon carcinogenesis. Methylenetetrahydrofolate reductase (MTHFR) catalyses the reduction of 5,10-methylene THF to 5-methyl THF. Methionine synthase (MS) catalyzes the transfer of the methyl base from 5-methyl THF to homocysteine to generate methionine; and 10-formyl THF is a cofactor for purine synthesis (Fang and Xiao 2003). Betaine, formed from oxidation of choline, can transfer one of its three methyl groups to homocysteine to produce methionine, but this pathway operates mainly in rodents (Lamprecht and Lipkin 2003). Folate depletion decreases DNA methylation (global and gene specific) in certain human and animals but not in others (Duthie 2010). Zn, Vitamins B6 and B12 serve as a co-enzyme in different reactions. SAM, S-adenosyl L-methionine; SAH, S-adenosyl homocysteine; TS, thymidylate synthase; DNMT's DNA methyltransferases.

Other food compounds that have been found to impact upon the epigenome through DNA methylation changes include polyphenols such as (-) epigallocatechin 3-galleate (EGCG) from green tea and genistein from soybean (Fang et al. 2003; Fang et al. 2007). Another group of dietary phenolics that have been investigated as food compounds with potential effects on DNA methylation are: catechin, epicatechin, quercetin, fisetin, myricetin, cafeic acid and chlorogenic acid (Lee et al. 2005; Lee and Zhu 2006). These studies have found reactivation of multiple genes in oesophageal cancer cells due to reversal of gene-specific CpG methylation at several loci, obtaining similar effects in HT-29 human colon cancer cells, PC3 prostate cancer and MCF-7 breast

cancer cell lines (Lee et al. 2005; Fang et al. 2007). Likewise, selenium is an important micronutrient engaged in the protection of colonic cells against a wide range of external and internal stressors (Rudolf et al. 2008) and in addition to its chemopreventive roles in the different stages of colorectal cancer attributable to its anti-oxidative and anti-inflammatory effects through the activity of selenoenzymes, selenium has also been found to be involved in the inhibition of human DNA methyltransferase 1 (DNMT1) (Davis and Uthus 2004).

Despite the variety of compounds studied so far the apparent common mechanisms by which all these compounds reverse DNA hypermethylation and reactivate methylation-silenced genes is thought to be through a direct inhibition of DNA methyltransferase (DNMT) activity and also by a decrease in the concentration of SAM and increased levels of SAH, which is a potent inhibitor of DNMT (Lee and Zhu 2006).

Modification of histone acetylation is another epigenetic mechanism whereby bioactive food components can influence gene expression and cancer susceptibility. In this respect diallyl disulfide, an organosulfur compound present in garlic, has been found to inhibit cell proliferation through the inhibition of HDAC activity, histone hyperacetylation and increase in $p21^{waf1/cip1}$ *in vitro* and *in vivo* (Druesne et al. 2004; Druesne-Pecollo et al. 2007). Isothiocyanates have also been found to exert similar actions; however they will be discussed in detail in chapter 3. Other dietary factors known to influence DNA methylation and cancer susceptibility can be found in numerous reviews (Ross 2003; Davis and Uthus 2004; Johnson and Belshaw 2008).

New data highlighting the role of isothiocyanates and selenium in regulating the genome machinery through epigenetic mechanisms, and other factors altering the expression of antioxidant selenoenzymes, will be presented and

discussed in the next chapters in the context of an *in vitro* model of colon cancer.

1.9 Proposed Investigation

Colon cancer is a disease of the colonic mucosa, caused by a time-dependent accumulation of genetic changes that deregulate the normal process of colonic tissue regeneration. Although a number of hereditary genetic mutations are attributed to high risk of colon cancer it is clear that the majority of colon cancers result from a complex interplay of diet, environment, genetic and epigenetic factors. However, how different dietary components, in partnership, regulate gene expression linked to colon pathology is relatively unknown. It has been suggested that the combination of phytochemicals and other nutrients in fruits and vegetables is crucial for their potential anticancer activities. However, as many responses linked to nutrient-modulated pathways may take place simultaneously within the carcinoma process, it is difficult to determine which is most important in dictating the overall biological response and hence the importance in determining the exact role of each compound that contributes to their possible health benefit protection and then proceed to determine synergy between nutrients that might enhance their functional properties.

Although the majority of studies suggest that selenium is effective for disease prevention the disparity of results obtained in selenium clinical trials indicates that a more focused approach to understand the mechanisms of different forms of selenium on antioxidant and anticancer activity is needed. Also, the fact that abundant experimental evidence has shown anticarcinogenic effect of selenium in individuals with apparently full selenoenzyme expression (Combs and Lu 2006) suggests that other mechanisms could be involved in chemoprevention by this mineral. In this respect, as well as analysing the antioxidant properties of selenium this study will identify if alterations in DNA methylation may be a potential mechanism whereby dietary selenium decreases colon carcinogenesis.

Interestingly, for isothiocyanates conflicting evidence exists regarding the structure-activity relationship (SAR). While some studies have shown that isothiocyanates with longer alkyl chains have greater chemopreventive potential (Morse et al. 1989; Morse et al. 1991) others suggest that synthetic isothiocyanates with longer alkyl chains and increased lipophilicity do not necessarily have enhanced chemopreventive potential (Son et al. 2000). Although a proper SAR will involve the study of different isothiocyanates structures, my goal in this respect will be to determine if small differences in ITC structures (e.g., the alkyl chain length between sulforaphane [C4] and iberin [C3]) induce the antioxidant enzymes GI-GPx and TrxR1 differently.

Moreover, specific studies using a wide variety of isothiocyanates *in vitro* and *in vivo* have shown that one of the primary mechanisms by which this group inhibit carcinogenesis is by activation of phase II enzymes (see Table 2), but only a few of these studies have addressed other important groups of antioxidant enzymes such as GI-GPx and TrxR1 that equally respond to transcriptional activation by ITCs through the Nrf2/ARE system, and how these selenoproteins respond to different forms of ITCs or selenium is less well understood. Furthermore, of these two compounds the former have been found to act epigenetically as histone deacetylase inhibitors (Ho et al. 2009), but there is a lack of evidence supporting a role at the DNA methylation level.

While most studies have evaluated the impact of single isothiocyanates or selenium compounds in the expression of selenoproteins, no studies have compared an inorganic and organic form of selenium in combination with different forms of isothiocyanates to cross-examine their role in gene expression with regard to antioxidant enzyme and DNA methylation changes for colon cancer prevention.

In an attempt to understand the direct effect of two isothiocyanates with different carbon chain lengths namely sulforaphane and iberin in combination with Se-methylselenocysteine (organic Se) and selenite (inorganic Se) this

project will aim to establish whether a combination of food components offer additive or synergistic chemoprotective effects on the regulation of two selenoenzymes (TrxR1 and GI-GPx) and DNA methylation using human colon adenocarcinoma Caco-2 cells as a model to understand the cellular mechanisms associated with antioxidant enzyme regulation and aberrant CpG island methylation.

The possibility that diet could influence the direction and extent of antioxidant enzyme expression together with epigenetic changes opens new ways for cancer prevention. Since it has been established that the chemopreventive efficacy of selenium depends on the chemical form in which it is administered (Ganther 1999), and in view of the fact that Se-methylselenocysteine has been found in animal and cell culture models to be the most effective anti-carcinogenic form of selenium (Ip et al. 1991), this chemical form will be employed in this study. Likewise, iberin, a sulfoxide analogue of sulforaphane is a naturally occurring member of the isothiocyanate family of cancer chemopreventive agents that might hold similar or more potent properties against tumour cells than its more widely studied analogue sulforaphane.

Finally, a combinatorial chemopreventive strategy that considers the chemical form and structure of different food compounds might help to unravel the relative contribution of various components of a plant-based diet to overall cancer reduction and provide insight into the potential synergy among essential nutrients and non-nutrients that may counteract the genetic and epigenetic alterations that initiate and sustain neoplasia.

Initially, this study will present work to evaluate the efficacy of these food compounds, either individually or in combination, on the expression of GI-GPx and TrxR1 (Chapter 2). Work to investigate effects of these compounds on global and gene-specific methylation patterns and epigenetic signals will be presented (Chapter 3), followed by an outline of the principles and challenges of applying CpG island microarrays in nutrition studies (Chapter 4). The work

concludes with a summary of the data presented in this thesis and priorities for future research (Chapter 5).

2

Modulation of the Expression of Antioxidant Enzymes TrxR1 and GI-GPx by Isothiocyanates and Selenium in Caco-2 Cells

2.0 INTRODUCTION

Several lines of evidence have implicated chronic inflammation as a key predisposing factor for tumour progression, suggesting that oxidative stress is likely to play an important role in this process (Colotta et al. 2009). Accumulation of reactive oxygen and nitrogen species (ROSN) generated by inflammatory cells is thought to be one of the major mechanisms by which chronic inflammation contributes to neoplastic transformation as well as many other diseases (Benz and Yau 2008). Both extracellular and intracellular antioxidative defence systems protect DNA from oxidative stress. Among these, various selenocysteine-containing proteins, so-called selenoproteins, have been demonstrated to confer protection from ROSN-related oxidative damage (Yoshiyuki et al. 2008).

Different forms of selenium supplementation, either as inorganic Se (sodium selenite) or as organic forms such as Se-methylselenocysteine may have different efficacies in cancer prevention. As mentioned in Chapter 1 cruciferous vegetables store glucosinolates but, in addition, they are also able to accumulate selenium in different forms. It has been shown that selenium-enriched broccoli is more effective than traditional broccoli or other selenium inorganic forms in protecting against chemically-induced mammary cancer and against aberrant crypt formation in Min mice (predisposed to neoplastic lesions associated with the *APC* gene in the intestine) (Finley et al. 2000; Finley et al. 2001; Davis et al. 2002). These findings and the observation that isothiocyanates are able to promote the induction of the selenoproteins thioredoxin reductase-1 (TrxR1) and gastrointestinal glutathione peroxidise (GI-GPx) brings selenium and isothiocyanates (ITCs) into an interesting focus to identify synergistic mechanisms of induction and unravel potential links between both dietary factors in the prevention of cancer through the induction of the antioxidant system that controls the cellular redox state, thereby protecting against oxidative damage.

2.1 MATERIALS AND METHODS

2.1.1 Materials

A human colon adenocarcinoma Caco-2 cell line was purchased from the American Type Culture Collection (Middlesex, UK). Cell culture media and supplements were purchased from Invitrogen (Paisley, Scotland), except for fetal bovine serum (FBS) obtained from Biosera, UK. Sulforaphane and iberin was purchased from LKT Laboratories (Alexis Biochemicals, UK) and Seleno-methyl-selenocysteine (SeMSC) from Eburon Organics (Belgium). Dimethyl sulfoxide (DMSO), sodium selenite, hydrogen peroxide (H_2O_2), RT-PCR primers and probes used, and all other chemicals were purchased from Sigma-Aldrich (UK), unless otherwise stated.

2.1.2 Methods

2.1.2.1 Cell Culture

Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), containing 4.5 g/L D-glucose and Non Essential Amino Acids (NEAA), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 1% (v/v) of L-glutamine (200 mM) and 1% (v/v) antibiotic solution consisting of penicillin (5000 units/ml), streptomycin (5000 µg/ml).

It is important to mention that most mammalian cell culture media require a certain concentration of serum, which contains selenium at a concentration that is high enough to maintain the expression of cellular glutathione peroxidises. The DMEM medium used in this study is a Se-free medium, however, under normal cell culture conditions FBS is a major source of selenium. The level of selenium in FBS was 13 µg/L (equivalent to a final concentration of 16 nM Se

when 10% of FBS is added to the cell culture medium, which is still considered as a selenium-deficient medium) as determined by Biosera (UK).

Cells were maintained in a controlled atmosphere with 5% CO_2 at 37ºC in a HERAcell® 150 CO_2 incubator. Cells were kept growing in Nunclon™-Δ flasks (75cm^2) and when confluence reached 80% cells were passaged following the standard operating procedures for subculture of adherent cell lines. Briefly, medium was extracted and monolayers were rinsed twice with 10 ml of sterilized Dulbecco's phosphate buffered saline (PBS), then 600 µl of trypsin/EDTA (0.25% trypsin, 1 mM EDTA) were added, rocking the flask to ensure that the entire monolayer was covered with the trypsin solution, leaving the flask at 37^{o}C for 3-6 minutes until the cells were detached. After this, cells were resuspended in 10 ml of fresh serum-containing medium to inactivate the trypsin, pipetting the cells up and down until the cells were dispersed into a single cell suspension. Cells were treated using a Class II Biological Safety Cabinet. All instruments used were sterilized (tips, pipettes) and the cabinet was carefully disinfected with TriGene 2% and gloves were always worn.

2.1.2.2 Cell Viability Assay to Determine the Cytotoxicity of ITCs and Se

Caco-2 cells were seeded in 96-well plates (BD Falcon™) in DMEM at a concentration of 3.0×10^3 cells in a final volume of 100 µl per well, and left to adhere to the plastic plates for 24 hours before they were treated with 100 µl of DMEM containing different concentrations of sulforaphane and iberin (ranging from 1 to 50 µM) or SeMSC and selenite (ranging from 0.2 to 200 µM), using five biological replicates per concentration. Isothiocyanate compounds were dissolved in DMSO keeping an equal final concentration of 0.05% in all treatments and controls. Selenium compounds were dissolved in Milli-Q water and filtered through a 0.2 µM syringe filter. All stock and working solutions were aliquoted and stored at -80°C. Different time points were used to

determine cell viability, also useful for long term experiments carried out for DNA methylation experiments described in Chapter 3. Cell viability was determined in four independent plates at 24, 48, 72 and 96 hours. For the last two plates, media containing food compounds were replaced after 48 hours to maintain a constant supply of ITCs and Se to the cells. Ten microliters of WST-1 (Roche, UK) reagent was added to each well to facilitate the reaction between mitochondrial dehydrogenase released from viable cells and tetrazolium salt of WST-1 reagent. The intensity of the coloured compound formed (formazan dye) was then quantified using a micro plate reader (BMG Labtech). Initially, the absorption was measured at different time points after the addition of WST-1 (e.g. 0.5, 1, 2, 4) to determine the most suitable incubation time, determining that 2 hours was the optimal time. The absorbance was measured at 450 nm, with the reference at 630 nm. Cell viability of treated cells was expressed as a percentage of control as follows: $(A_{450}$ nm - A_{630}nm)sample/(A_{450} nm - A_{630} nm)control × 100. The IC_{50} was determined using CalcuSyn software Version 2.0 (Biosoft, Cambridge, UK).

2.1.2.3 Induction of TrxR1 and GI-GPx by Isothiocyanates and Selenium

2.1.2.3.1 Cell Culture

Caco-2 cells were cultured following the same conditions described in section 2.1.2.1 Cells were seeded in six-well plates at 6×10^4 cells/well in a final volume of 3 ml and were supplemented with iberin and/or SeMSC when confluence reached ~70% (usually after 3 days). For time course experiments all different groups were treated at the same time and harvested at appropriate intervals using 3 biological replicates per group. All treatments and controls contained a final DMSO concentration of 0.05%.

2.1.2.3.2 RNA Isolation

Total RNA was extracted from Caco-2 cells using GenElute™ Total Mammalian RNA kit (Sigma, UK) according to the manufacturer's instruction and then stored at -80ºC. The NanoDrop spectrophotometer (Labtech International, UK) was used to quantify RNA concentration and purity by absorbance measurement at 260 and 280 nm.

2.1.2.3.3 Reverse Transcription

First strand cDNA was synthesised from total RNA. Briefly, 1μg of total RNA was incubated with 1 μl of qScript cDNA SuperMix (5×) (Quanta BioSciences, USA), which contains optimised concentrations of $MgCl_2$, dNTPs, recombinant RNase inhibitor protein, qScript reverse transcriptase, random primers and oligo(dT) primer. All components were mixed in a 96-well plate, vortexed and centrifuged in a final volume of 10 or 20 μl and incubated in a PCR Thermal Cycler (Applied Biosystem, Warrington, UK) using the following cycle: 5 min at 25ºC followed by 30 min at 42ºC and 5 min at 85ºC. After the cycle was completed, samples were diluted and stored at -20ºC for later use. Always, a No Template Control (NTC) reaction was included in every PCR assay to rule out reagent or water contamination

2.1.2.3.4 Real-Time PCR

Quantitative real time PCR was performed to measure the mRNA levels of target genes. TrxR1 and GI-GPx mRNA quantification was determined by TaqMan using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Primers and the fluorogenic TaqMan probes were designed using Primer Express® Software (Applied Biosystem, UK) based on the human *TrxR1* and *GI-GPx* sequences (Table 2.1). The probes were labelled with a 5´ reporter dye, FAM (6-carboxyfluoroscein) and 3´

quencher dye, TAMRA (6-carboxytetramethylrhodamine). Real-time PCR reactions were carried out in a 96-well plate in a total volume of 25 µl per well consisting of 10 ng of cDNA for target genes (*GI-GPx* and *TrxR1*) or 0.05 ng for the housekeeping gene 18S rRNA plus 10 µl Precision^{TM} MasterMix (Primer Design, UK). Primers and probes were added as follow: 100 nM probe, 200 nM forward and 300 nM reverse primers to amplify *TrxR1*. For the *GI-GPx* and 18S rRNA genes 100 nM probe, 200 nM forward and 200 nM of reverse primers were used (Table 2.1). Samples were run using a 10 min hot start at 95ºC, followed by 45 cycles of denaturing at 95 ºC for 15 s and annealing/extension at 60ºC for 60 s. Reactions were carried out using two technical replicates for every biological replicate. As a quality control procedure an NTC reaction from the cDNA conversion (section 2.1.2.3.3) was run together with an additional NTC from the reagents and water used for the RT-PCR reaction to rule out contamination.

Data were normalised against an invariant endogenous control, 18S ribosomal RNA. The threshold cycle number (Ct) obtained was converted into fold of relative induction using the ΔΔCt method (Livak and Schmittgen 2001). To test if this method was appropriate for the experimental conditions used, the real-time PCR efficiency (E) of the target genes was calculated and compared with that of the housekeeping gene by running standard curves and using the following equation: $E = (10^{-1/slope} - 1) \times 100$. The difference of efficiency between the genes of interest (*GI-GPx* and *TrxR1*) and endogenous control (18S rRNA) was less than 5% (McPherson and Moller 2000).

Table 2.1 Primers and Probes Sequences Used for Real-Time PCR

Gene		Sequence (5' – 3')
TrxR1	Forward	CCACTGGTGAAAGACCACGTT
	Reverse	AGGAGAAAAGATCATCACTGCTGAT
	Probe	CAGTATTCTTTGTCACCAGGGATGCCCA
GI-GPx	Forward	CACACAGATCTCCTACTCCATCCA
	Reverse	GGTCCAGCAGTGTCTCCTGAA
	Probe	CATGCTGCATCCTAAGGCTCCTCAGG
18S rRNA	Forward	GGCTCATTAAATCAGTTATGGTTCCT
	Reverse	GTATTAGCTCTAGAATTACCACAGTTATCCA
	Probe	TGGTCGCTCGCTCCTCTCCCA

The Real-Time PCR system is based on the detection and quantitation of a fluorescent reporter signal. As the fluorescent reporter only fluoresces when associated with the amplicon, the increase in recorded fluorescent signal during amplification is in direct proportion to the amount of amplification product in the reaction, making it possible to monitor the PCR reaction during the exponential phase. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescent is observed. A fixed fluorescent threshold is set significantly above the baseline that can be altered by the operator. The parameter Ct (threshold cycle) is defined as the cycle number at which the fluorescent emission exceeds the fixed threshold (Wong and Medrano 2005; Nolan et al. 2006).

2.1.2.3.5 Western Blot Analysis

After treating Caco-2 cells with sulforaphane or iberin (using concentrations ranging from 6 to 10 µM) and selenite or SeMSC (using concentrations ranging from 25 to 200 nM) or these compounds in combination (ITCs+Se), proteins were extracted at different incubation times (8, 24, 48 h) by washing cells twice with ice-cold phosphate-buffered saline (PBS) and treating them for

2 min with 100 µl of ice-cold cell lysis buffer containing 1% Nonidet P-40, Tris-EDTA buffer pH 8.0 (20 mM Tris, 2 mM EDTA), 150 mM NaCl, 10% glycerol and 1 × protease mixture (Complete Mini, Roche), before cells were collected by the use of a rubber policeman. The lysates were thereafter placed in an Eppendorf tube and incubated on ice for 15 min (vortexing the samples 3 times at full speed during incubation) with a final centrifugation step for 10 min at 13,000 × g to collect the supernatants. Protein concentrations were measured using the 96-well plate Bradford protein assay. Briefly, a standard solution of bovine serum albumin (2 mg/ml) was used to generate a standard curve using a concentration that ranged between 0.125 – 2 mg/ml. For protein samples with unknown concentration duplicates were prepared using a 1:10 dilution scheme (ensuring that the concentration remained within the linear range) by mixing 5 µl of the protein sample with 250 µl of the Bradford reagent and leaving the samples to incubate at room temperature (RT) for 15 min, using as a blank 5 µl of 1:10 diluted buffer. Absorbance was measured at 595 nm using a microplate reader (BMG Labtech) and the protein concentration of the unknown samples was determined using the Omega data analysis software by comparing the net A_{595} values against the standard curve.

Equivalent amounts of protein (40 µg for TrxR1 and 60 µg for GI-GPx) were mixed with loading buffer (0.625M Tris, 2% SDS, 10% glycerol, 20mM DTT and bromophenol blue) and heated at 95 ºC for 5 min to be size fractioned in 12.5% SDS-polyacrylamide gel electrophoresis at 25 mA for 1 gel or 35 mA for two gels prior to transfer to Immun-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a Trans-Blot semi-dry transfer system (Bio-Rad) at 15 V/gel for 1 hour. To verify that the proteins were transferred efficiently from the gel to the membrane, the gel was stained with Instant Blue Coomassie reagent (Expedeon, UK). The membrane was blocked for 1 h at RT with 5% (w/v) non-fat milk in PBST (1×PBS, 0.5% Tween-20), followed by overnight incubation with primary antibody at 4°C with gentle agitation using antibodies against TrxR1 (rabbit polyclonal IgG, Santa Cruz), GI-GPx (rabbit anti-human GI-GPx kindly donated by Prof. Regina Brigelius-Flohé) and beta-actin (goat

polyclonal IgG, Santa Cruz). The dilutions used for TrxR1, GI-GPx and β-actin antibodies were 1:1000, 1:2000 and 1:20000 with 5% (w/v) non-fat milk diluted in PBST respectively. Membranes were washed three times for 10 min each with PBST and incubated for 1 hour at RT with permanent agitation and appropriate secondary antibody (goat anti-rabbit IgG Santa Cruz) at a final dilution of 1:20000 with 5% non-fat milk in PBST, using the same dilution for β-actin but rabbit anti-goat IgG instead (Santa Cruz). Then, the membranes were washed three times with PBST for 10 min each, and developed using an enhanced chemiluminescence (ECL) system (Amershan, GE Healthcare, UK), visualized using a LAS-3000 Fujifilm intelligent dark box. The illuminated bands were detected and the image captured using Image Reader LAS-3000 software. Densitometric analysis of the Western blot was performed using Quantity One® Ver. 4.6.3 basic software (Bio-Rad Laboratories, UK). The volume rectangular tool was used to create a volume box around an image and the average intensity of the pixels in the background volumes was subtracted by using the global background subtraction application tool in the Quantity One® software. Results were normalised against β-actin to correct for protein loading, and the protein expression from the different treatments was calculated relative to control.

2.1.2.4 Role of Nrf2 in Isothiocyanate- and/or Selenium-mediated Antioxidant Enzyme Expression

2.1.2.4.1 *Nrf2* siRNA Interference Assay

Nrf2 siRNA (siRNA ID 115764) and scrambled siRNA (Silencer® Select Negative Control #1) were obtained from Applied Biosystems and used to inhibit *Nrf2*. Before purchasing the *Nrf2* siRNA, the target sequences obtained from the company (Sense strand: 5'-CCUUAUAUCUCGAAGUUUUtt-3'; antisense strand: 5'-AAAACUUCGAGAUAAGGtg-3') were checked using the Entrez Gene database provided by NCBI to make sure that the siRNA

sequence targets the different *Nrf2* splice variants as the RT-PCR primers and probe were designed to identify all known transcripts for this gene (see section 2.1.2.4.2). Transfection of siRNA was performed using Lipofectamine 2000 according to manufacturer's protocol (Invitrogen). Briefly, the day before transfection cells were seeded at 5×10^4 cells/well in a 24-well plate in 500 µl of growth medium without antibiotics. On the day of transfection, 3 µM of siRNA and 1.5 µl of Lipofectamine were diluted separately in 50 µl culture medium without FBS, and incubated for 5 min at RT. After the 5 min incubation, the diluted siRNA was mixed with diluted Lipofectamine 2000 (total volume=100 µl) and incubated for a further 20 min at RT. Then, the transfection complexes were added drop-wise to each well containing cells and medium to obtain a final volume of 600 µl to achieve a final siRNA concentration of 30 nM, mixing gently by rocking the plate back and forth. For siRNA experiments three controls were performed in which a) cells received no Lipofectamine 2000 and no siRNA (untransfected cells control), b) cells received only Lipofectamine 2000 (mock transfection control), and c) cells were transfected with Lipofectamine 2000 and 30 nM (final concentration) of a scrambled siRNA sequence (negative control). To study the impact of isothiocyanates (ITCs) and/or selenium (Se) on *TrxR1* and *GI-GPx* expression when *Nrf2* was knocked down, wells used to compare basal levels of expression of these genes in the presence of ITCs and/or Se (without *Nrf2* repression) were also transfected with scrambled siRNA to mirror conditions used with siRNA *Nrf2* transfected cells, allowing a direct comparison with the rest of the treatments. Transfected cells were incubated at 37 ºC for 24 h before media were removed and cells washed with 600 µl of PBS, and treatments of iberin (6 µM), sulforaphane (6 µM) and selenite (200 nM) or combinations applied in serum-containing DMEM media without antibiotics for 12 hours. RNA was then extracted following steps on section 2.1.2.3.2

To obtain the highest transfection efficiency and low non-specific effects the transfection conditions were optimised first before the experiment was carried out by using different volumes of Lipofectamine 2000 (0.5, 1.0 and 1.5 µl)

while keeping the siRNA quantity (30nM) fixed, selecting 1.5 µl of Lipofectamine 2000 as the volume that offered the best performance.

2.1.2.4.2 Quantification of Nrf2 by Real-Time PCR

Nrf2 primers were constructed to determine its level of expression after silencing. This was done by identifying first its sequence at http://www.ncbi.nlm.nih.gov/sites/gene according to the appropriate source organism: *Homo sapiens*. The primers and probes (Table 2.2) were designed to amplify the different transcript variants of *Nrf2*. In order to do this a common region between the different splice variants identified (NM_006164.3, NM_001145412.1 and NM_001145413.1) was found by aligning the FASTA format sequences using the online tool at http://align.genome.jp/ (clicking the 'Slow/Accurate' and 'DNA' radio buttons). Then, this common coding region was used as the target for Primer Express® software to design primers and dye-labelled probes that recognize all the splice variants. After the primers were designed their sequences were interrogated by using the Basic Local Alignment Search Tool (BLAST) from the NCBI website http://www.ncbi.nlm.nih.gov/tools/primer-blast/ to search for homology with other genes, identifying none. The RT-PCR set up was similar to that specified in section 2.1.2.3.4. However, the concentration of primers and probes optimised for this gene to match the housekeeping gene efficiency corresponded to 100 nM probe, 200 nM forward and 150 nM of reverse primers.

Table 2.2 Primers and Probes Sequences Used for RT-PCR

Gene		Sequence (5' – 3')
Nrf2	Forward	TGGTACAACCCTTGTCACCATC
	Reverse	AATTCTTTCTCTGGTGTGTTCT
	Probe	AGCACTCACGTGCATGATGCCCA

2.1.2.4.3 Nuclear and Cytoplasmic Protein Extraction

Nuclear and cytoplasmic protein extracts from Caco-2 cells were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, Caco-2 cells were seeded at 4×10^5 cells in 10-cm dishes and treated when confluence reached 80% (usually after 4 days) with vehicle DMSO and SeMSC (200nM), iberin (12 µM) or combinations of these compounds for 8 and 24 hours. For the last time point iberin (6 µM) was also included in combination with SeMSC. At the experimental time points, media were removed, and cells were washed with 10ml of ice cold PBS, then 500 µl of PBS was added to remove the cells by the use of a rubber policeman. Cell were then centrifuged at 500×*g* for 3 min at 4°C, supernatant was removed and cells were resuspended in Cytoplasmic Extraction Reagent I buffer (CER I) (containing protease inhibitor) by vortexing the tube for 15 seconds followed by incubation on ice for 10 minutes. Then, CER II buffer was added to the samples, vortexed, and centrifuged at 16000×*g* for 5 minutes to separate the supernatant, which contained the cytosolic proteins. The pellet was resuspended in Nuclear Extraction Reagent buffer (NER)r (containing protease inhibitor) and vortexed every 10 minutes for 40 minutes, and then centrifuged at 16000×*g* for 10 minutes to separate the supernatant, which contained the nuclear proteins. All the extracts were stored at -80°C until use. Protein concentrations of cytoplasmic and nuclear extracts were determined by the Bradford method.

For detection of Nrf2 by Western blotting, the same steps described in section 2.1.2.3.5 were followed. However, *Nrf2* bands in the nuclear and cytosolic fraction were detected using two different rabbit polyclonal anti-*Nrf2* antibodies, namely H-300 and C-20 respectively (Santa Cruz, CA), using a 1:1000 dilution. After an overnight incubation with the primary antibody the membranes were washed three times with PBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz, CA), using a 1:5000 dilution. Equal loading of Western blots was determined by the

use of rabbit polyclonal antibody SAM 68 and goat polyclonal antibody β-Actin (Santa Cruz, CA) for the nuclear and cytosolic fraction respectively, using the appropriate secondary antibody against the species in which the primary antibody was raised.

2.1.2.5 Effects of Hydrogen Peroxide on Caco-2 Cells Pre-treated with Isothiocyanates and/or Selenium Compounds.

2.1.2.5.1 Cytotoxicity of Hydrogen Peroxide

Before determining the protection by ITCs and/or Se compounds in Caco-2 cells challenged with oxidative stress, cells were treated in a dose-dependent manner with the oxidant hydrogen peroxide (H_2O_2) to determine its effect on cells viability. Furthermore, to determine the effect of increasing concentration of H_2O_2 on cell viability when Caco-2 cells were pre-treated with dietary constituents, to identify the best concentration to use under these conditions, an additional plate pre-treated with selenite was set up before increasing concentrations of H_2O_2 were added. To achieve this, two 96-well plates were seeded at 7×10^3 cells/well using 6 biological replicates per treatment in a final volume of 100 µl DMEM and left to grow until confluence reached about 50-60% (usually after 48 h). Then, media from both plates were removed before one of the 96-well plates was supplemented with 100 µl of a stock solution of selenite diluted with DMEM to 50 nM and the second plate supplemented with 100 µl of media containing the vehicle (water). After 24 h, H_2O_2 stock solutions (dissolved in water) and diluted with serum-free DMEM (containing only 1% of L-Glutamine and 1% penicillin/streptomycin) were prepared before culture media were removed from both plates, which were then incubated with 100 µl of media containing the vehicle (water) or increasing concentrations of H_2O_2 ranging from 600 to 1200 µM, for the plate treated with selenium, or ranging from 200 to 800 µM, for the plate without selenium, for a period of 24

h prior to the addition of 10 µl of WST-1 to determine cell cytotoxicity (see section 2.1.2.2).

2.1.2.5.2 ITC- and/or Se-mediated Cytoprotection in Caco-2 Cells

The protective effect of ITCs and/or Se against H_2O_2-induced cytotoxicity was also investigated. Caco-2 cells (7×10^3 cells/well in a 96-well plate) were grown for 48 hours before media was removed and cells were treated with 100 µl of DMEM containing the vehicle (DMSO), SFN (6 µM), selenite (50 nM), and a combination of ITCs+Se to evaluate its synergistic effect using 12 replicates per treatment. After 24 hours stock solution of H_2O_2 were diluted with serum-free media to obtain a final concentration of 500 µM before media was removed from the cells and 6 replicates were treated with 100 µl of H_2O_2 and the other 6 with an equal amount of the vehicle DMSO (0.05%) to compare the viability of cells treated only with SFN and/or selenite against that generated by cells treated with the food compounds plus the oxidants for 24 hours. After 24 h of incubation was completed 10µl of WST-1 was added to the media and cell viability was measured at 30 and 60 min following steps on section 2.1.2.2.

2.1.2.6 Role of *TrxR1* and *GI-GPx* in the ITC- and/or Se-mediated Cytoprotection in Caco-2 cells

2.1.2.6.1 *TrxR1*/*GI-GPx* siRNA Optimisation Assay

To achieve the best results in siRNA transfection of adherent cells, the amount of siRNA and the ratio of HiPerFect Transfection reagent to siRNA were optimised by combining 1, 5, 25 and 50 nM of siRNA with different

concentrations of HiPerFect (1.5, 3.0 and 4.5 µl) using a 24-well plate format, followed by optimisation of a single and double knockdown approach, selecting 25 nM siRNA and 3.5 µl of HiPerFect for the former (for the experiments described in section 2.1.2.6.2 this amount was adapted to a 96-well plate format). Silencing was monitored at the mRNA level by real-time PCR following steps described in section 2.1.2.3.4 and using the same primers and probes sequences shown in Table 2.1.

2.1.2.6.2 Cell Viability Effects of *TrxR1* and/or *GI-GPx* Knockdown in Caco-2 cells

A single and double knockdown approach was used to investigate the effect of the selenoproteins TrxR1 and GI-GPx in cell protection when cells were pre-treated with SFN+Selenite and challenged with H_2O_2. *TrxR1* siRNA (Target sequence: CTGCAAGACTCTCGAAATTAT), *GI-GPx* siRNA (Target sequence: AACCCTCTGGTTGGTGATTCA) and AllStars negative control siRNA were obtained from Qiagen. Transfection of siRNA was performed using HiPerFect (Qiagen) using the Fast-Forward protocol for adherent cells provided by the manufacturer. Briefly, on the day of transfection Caco-2 cells were seeded at 1.8×10^4 cells/well of a 96-well plate in 170 µl of 10% FBS DMEM medium (containing 1% of L-Glutamine and 1% penicillin/streptomycin) and were incubated under normal growth conditions until the transfection complex was ready to be dispensed. In order to guarantee that the single and double knockdown treatments contained an identical final concentration of siRNA, the former was mixed with AllStars negative control siRNA (scrambled siRNA). For the experimental set up the following treatment groups were prepared: a) cells received only AllStars siRNA (50 nM) (control group), b) cells received AllStars siRNA and SFN+Se (second control group without *TrxR1* or *GI-GPx* siRNA), c) Cells received *TrxR1* siRNA + AllStars siRNA and SFN+Se (*TrxR1* single knockdown), d) Cells received *GI-GPx* siRNA + AllStars siRNA and SFN+Se (*GI-GPx* single knockdown), e)

Cells received *TrxR1* siRNA + *GI-GPx* siRNA + SFN+Se (*TrxR1*/*GI-GPx* double knockdown). For every experimental group 12 biological replicates were prepared.

To prepare the transfection complex of the single knockdown treatments, stock solutions of *TrxR1* or *GI-GPx* siRNA and AllStars negative control siRNA were diluted in 30 µl of plain DMEM (to give a final siRNA concentration of 50 nM after adding complexes to cells seeded in 170 µl DMEM). For the double KO a similar approach was followed to obtain a final concentration of 25 nM of *TrxR1* plus 25 nM of *GI-GPx* (50 nM in total) when the transfection complex (30 µl) was added to the cells. Likewise, the control groups were prepared by adding 50 nM of AllStars negative control siRNA. Once every siRNA was diluted in 30 µl of the plain culture medium, 0.875 µl of HiPerFect was added, mixed by vortexing and incubated for 5-10 min at room temperature to allow the formation of the transfection complexes. Subsequently, the complexes were added drop-wise onto the cells, swirling the plate gently to ensure its uniform distribution, and then cells were placed in the incubator. After 24 h the media were removed and cells were treated with 100 µl of DMEM containing DMSO (control group) or SFN (6 µM) plus selenite (50 nM). After 24 h the medium was removed again and serum-free medium containing 400 µM of H_2O_2 was added only to 6 biological replicates per every treatment group. For the remaining 6 wells, only serum-free medium containing the vehicle (water) was added (this group served as the control for every treatment). After 24 h of incubation with H_2O_2 or vehicle 10µl of WST-1 was added to the media and cell viability was measured at 30 and 60 min following steps described in section 2.1.2.2.

2.1.3 Statistical Methods

Statistical analyses were carried out using the SPSS 16 statistical program. Results are expressed as means ± SD. Statistical comparisons were made using Student's *t*-test and one-way ANOVA with Dunnett's *post hoc* test when

treatment samples were compared only against the control group and with Bonferroni's *post hoc* test when comparisons between groups were made.

2.2 RESULTS AND DISCUSSION

2.2.1 Effect of Isothiocyanates and Selenium on Cell Survival

To ensure that doses used were not toxic to the cells, Caco-2 cells were exposed to increasing concentrations of ITCs (ranging from 1 to 50 µM) and selenium (ranging from 0.2 to 200 µM) to evaluate cell growth. A profile

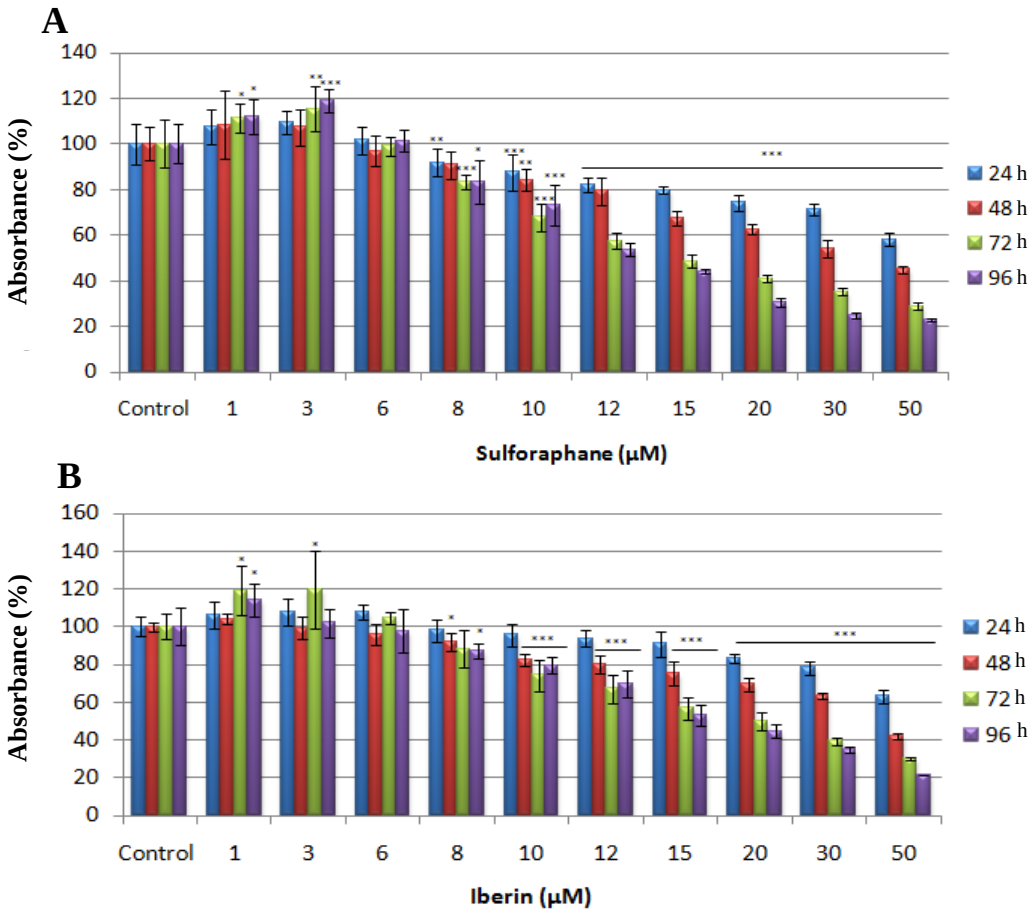


Figure 2.1 The effect of (A) sulforaphane and (B) iberin on Caco-2 cells as measured by cell metabolic assay WST-1. Caco-2 cells were treated for up to 96 h with DMSO (control) or with the indicated concentrations of selenium, and cell cytotoxicity was evaluated. Data (optical density) represent the mean ± SD of five replicates expressed as percentage of control. Significant differences from control are indicated (**P*<0.05; ***P*<0.01; ****P*<0.001). Lines drawn on the graph indicates that all the bars included within the line are significantly different different from their corresponding control group.

comparison of cell growth inhibition after ITC exposure for up to 96 h revealed obvious differences between short and longer exposure times particularly with higher ITC concentrations. The viability of Caco-2 cells supplemented either with sulforaphane or iberin was similar for both ITCs and cell proliferation was not affected by ITCs at the low concentration range (1-8 µM). However, concentrations of sulforaphane and iberin above 10 µM reduced significantly the viability of cells in a time- and dose-dependent manner, particularly after 48 h (Figure 2.1). In general, the IC_{50} value obtained for both ITCs indicates that cells are more sensitive to sulforaphane than iberin particularly after 24 h treatment, with a similar value thereafter (Table 2.3). These results are in agreement with published data (using different cytotoxicity assays), showing that treatment of colon cancer cells (Caco-2, SW620, HT-29,) with ITC concentrations >10 µM caused a significant decrease in cell viability (Gamet-Payrastre et al. 2000; Jakubikova et al. 2006; Andelova et al. 2007; Harris and Jeffery 2008). However, our results differ markedly from those obtained by Traka et el., who report an IC_{50} value of 85 µM for Caco-2 cells after 24 hours' treatment with sulforaphane. This discrepancy might be explained as a result of the different metabolic-dye assay (MTT) employed by them to determine cell viability (Traka et al. 2005).

Table 2.3 IC_{50} values of sulforaphane and iberin obtained after treating Caco-2 cells from 24 to 96 h

Time (h)	IC_{50} (µM)*	
	Sulforaphane ± SD	Iberin ± SD
24	35±2.5	45±2.5
48	27±2.3	29±2.2
72	20±2.7	22±3.1
96	18±2.9	20±2.8

*IC_{50} measured using CalcuSyn software (Biosoft, Cambridge, UK). Each point represents the mean ± SD of five replicates

Regarding the cell response after selenium treatments, Figure 2.2.A shows a sustained increase in the percentage of cell survival when cells were

supplemented with increasing concentrations of selenite ranging from 0.2 to 25 µM for 72 hours. This trace mineral also produced a gradual decrease after 24 hours with treatment ≥50 µM, with a sudden decrease in cell viability after cells were supplemented with ≥10 µM of selenite for 96 h. The effect of SeMSC on cell viability (Figure 2.2.B) was comparable to that obtained for selenite and no toxicity was observed with concentrations ≤ 10 µM when cells were treated for up to 72 h. Treatment with SeMSC only caused a decrease in cell viability when cells were treated with concentrations above 100 µM for 72 or 96 hours. The effective cytotoxic concentrations of selenite and SeMSC that inhibited 50% of cell growth are shown in Table 2.4.

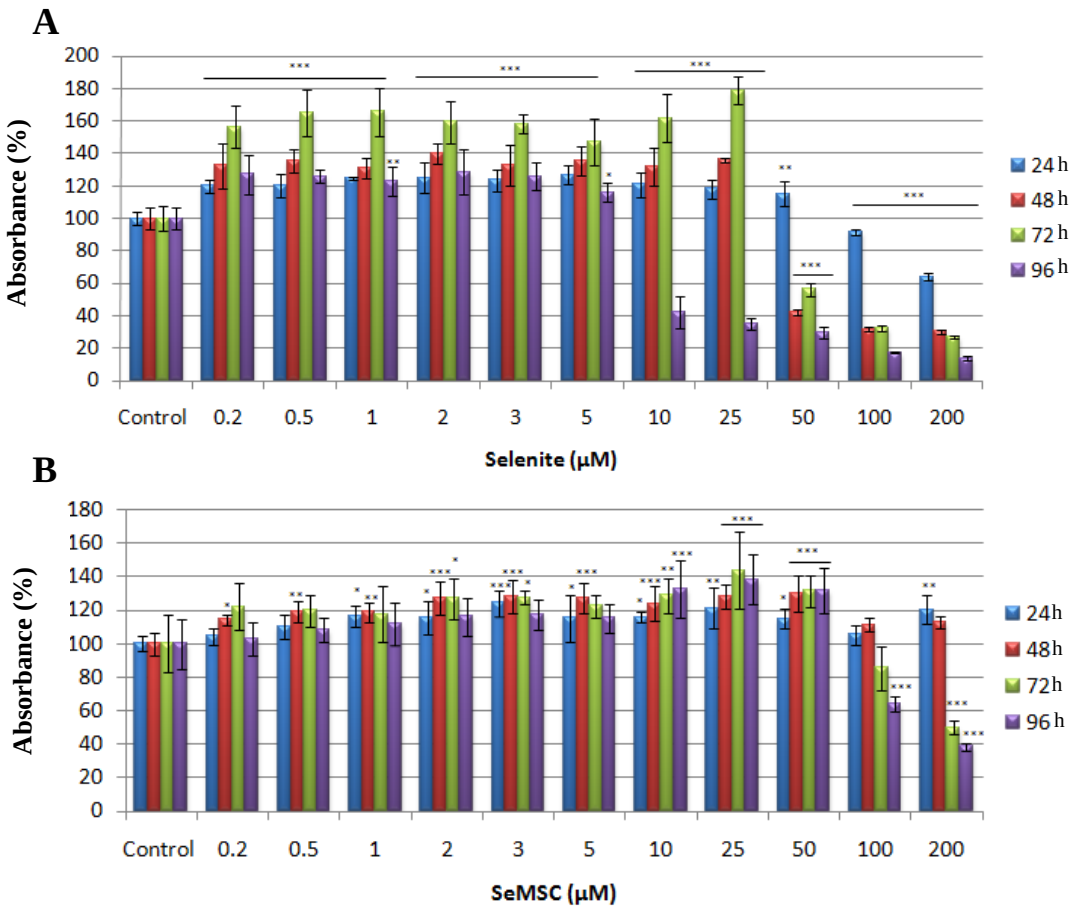


Figure 2.2 The effect of (A) selenite and (B) SeMSC on Caco-2 cells as measured by cell metabolic assay WST-1. Caco-2 cells were treated for up to 96 h with DMSO (control) or with the indicated concentrations of selenium, and cell cytotoxicity was evaluated. Data (optical density) represent the mean ± SD of five replicates expressed as percentage of control. Significant differences from control are indicated (**P*<0.05; ***P*<0.01; ****P*<0.001). Lines drawn on the graph indicates that all the bars included within the line are significantly different different from their corresponding control group.

In general, the available literature shows that cell culture models show a biphasic proliferation response to selenium, which has a stimulatory effect in the nanomolar to micromolar range, and a strong inhibitory effect at higher concentrations. However, when analysing the selenium literature for cell viability studies it is important to keep in mind the form of selenium used, the level of selenium to which cells were exposed, the cell type and the cytotoxicity assay used. For instance, Shroterova and colleagues (2009) showed that after comparing the toxicity of three common forms of selenium (selenite, selenomethionine and SeMSC) using five commonly employed cytotoxicity assays (WST-1, XTT, MTT, Brillian Blue and Neutral red assay) in three different colon cancer lines (HT-29, SW480 and SW620) and showed some differences in the sensitivity of the different cell lines to selenium treatment. However, the major observed differences resulted from the use of different cytotoxicity assays. Moreover, after using the WST-1 assay the viability of neither HT-29 nor SW620 cell lines was affected by selenite concentrations of up to 256 µM after 48 h treatment (Schröterová et al. 2009). Conversely, SeMSC was found to decrease cell viability by 55 and 40% in HT-29 and SW620 respectively at 256 µM (Schröterová et al. 2009). These findings were not consistent with the earlier literature, which suggests that selenite is more toxic than SeMSC (Kim et al. 2001), but different colon cancer cell types represent differing stages of the carcinogenesis process (HT-29 represents cells isolated from primary adenocarcinoma of colon grade 1, whereas SW620 correspond to a cell line isolated from metastasis of primary adenocarcinoma) and these cell types might be sensitised differently to various forms of selenium. For instance, a comparison of the IC_{50} value in different cancer cell lines (MCF-7, UACC-375, HT-29, DU-145, A-549) using selenomethionine found that different levels were required to cause 50% growth inhibition, identifying an IC_{50} value of 130 µM in the human colon cancer cell line HT-29 in comparison with the rest, which required less than 40 µM (Redman et al. 1998).

Also, in addition to assessing cell viability in response to individual ITCs and selenium, an experiment was set up using increasing doses of iberin (from 6 to 12 µM) plus SeMSC (from 0.2 to 1 µM) for up to 72 hours to identify changes in cell cytotoxicity when both compounds were added together. No levels below 95% of the control were detected in any of the treatments (data not shown).

Table 2.4 IC_{50} values of selenite and SeMSC obtained after treating Caco-2 cells from 24 to 96 h

Time (h)	IC_{50} (µM) *	
	Selenite ± SD	SeMSC ± SD
24	383±2.2	---
48	101±3.4	---
72	103±3.5	178±4.9
96	38±1.8	155±5.3

*IC_{50} measured using CalcuSyn software (Biosoft, Cambridge, UK). Each point represents the mean ± SD of five replicates

As shown in this study selenium was observed to promote an increase in cell viability. This observation is in agreement with previous data, where an increase in cell viability after selenite treatment at concentrations in the nanomolar to micromolar range has been demonstrated in human sarcoma (HT1080) or colon cancer (SW620) cell lines. In contrast no increase in viability was measured in the colon cancer cell HCT116 (Yoon et al. 2001; Schroeder et al. 2004). Also, HL-60 cells treated with ~10 µM of SeMSC were observed to have increased cell viability compared to controls, but treatment above this concentration promoted cytotoxicity (Kim et al. 2001). The mechanisms by which selenium promotes cell growth is thought to depend on the insulin-like action of Se including: increasing glucose uptake and ATP generation through the activation of glycolysis, antiapoptotic Bcl-2 protein up-regulation, maintenance of mitochondrial membrane potential, stimulation of fatty acid synthesis and pentose phosphate pathway activity (Yoon et al. 2002; Zeng and Combs Jr 2008).

Contrary to promoting cell growth, the increased toxicity that is usually observed for selenite in comparison to monomethylated selenium forms is due to the fact that selenite causes growth inhibition due to DNA single-strand breaks (a genotoxic effect), and increases cell death as a result of both necrosis and apoptosis. On the other hand, growth inhibition by a monomethylated selenium form such as SeMSC, is due to a decrease in cell proliferation and an increase in cell death in which apoptosis is the predominant mechanism, without involvement of DNA single-strand breaks (Ip et al. 2002). In general, the mechanism for the toxic effect of selenium at high levels has been suggested to be due to its ability to catalyse the oxidation of thiols and concurrent generation of ROS, which can damage cellular components by lipid peroxidation (Spallholz 1994). In fact, sodium selenite has been reported to generate oxidative stress in various cell models including colon cancer cells (Drake 2006; Kim et al. 2007; Xiang et al. 2009; Králová et al. 2010). In this respect, selenite, diselenides and the oxidation products of H_2Se, selenium dioxide, for example, can each react with glutathione (GSH) to produce the selenolate ion (RSe^-). In the presence of GSH and molecular oxygen, RSe^- can cycle continuously to generate $O_2^{\bullet -}$ and H_2O_2, which is thought to be the basis of Se-toxicity (Combs and Lu 2006).

At low doses, Se functions as an essential component of SeCys in several specific selenoproteins and promotes cell proliferation. At higher doses, but still not toxic, Se can reduce cancer risk (Zeng and Combs Jr 2008). SeMSC at a concentration of 50 µM induces apoptosis via ROS production in HL-60 cells (Jung et al. 2001), whereas in the nanomolar to micromolar range it confers a significant protection against an oxidative insult (Cuello et al. 2007).

Therefore, based on our results and taking into consideration other studies concentrations of ITCs and selenium compounds used for further experiments ranged from 3 to 12 µM for ITCs and 25 to 200 nM for selenium compounds. ITC concentrations in this range could be achieved in human plasma by

consumption of ITC-rich vegetables or high glucosinolate broccoli (Ye et al. 2002; Gasper et al. 2007).

2.2.2 Up-regulation of TrxR1 and GI-GPx by Isothiocyanates and Selenium Compounds

In the present study, the effects of isothiocyanates and selenium on *TrxR1* and *GI-GPx* gene expression in Caco-2 cells were evaluated. Time course experiments were conducted on the expression of these two important redox-modulating enzymes that have been shown to play an important role during

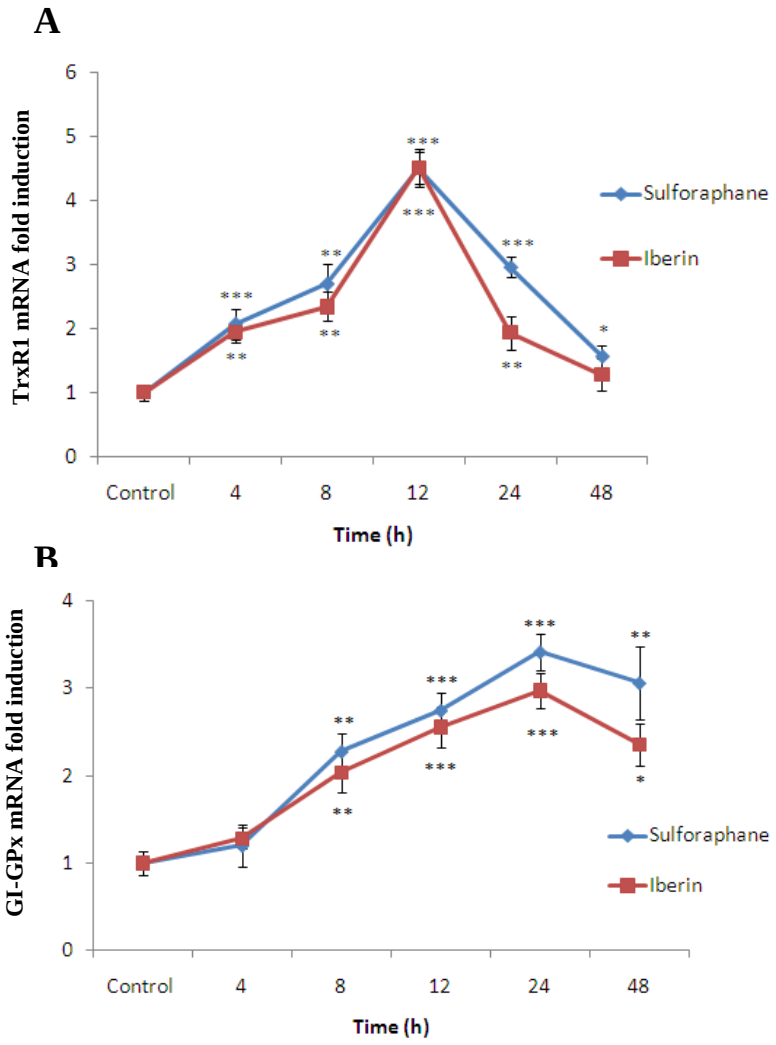


Figure 2.3 Time course of isothiocyanate-induced A) *TrxR1* and B) *GI-GPx* mRNA. Caco-2 cells were exposed to DMSO (control) or 6 µM of sulforaphane or iberin for different times to evaluate gene expression. Results are mean ± SD of triplicate samples normalized against 18S rRNA. Significant differences from control are indicated (**P*<0.05; ***P*<0.01; ****P*<0.001)

inflammation and cancer (Mustacich and Powis 2000; Chu et al. 2004). An initial experiment was carried out after exposing the cells to 6 μM of sulforaphane and iberin for 4, 8, 12, 24 and 48 h to provide information about the regulation of mRNA over time. The results showed a significant increase after sulforaphane or iberin treatment in a time dependant manner in the expression of TrxR1 and GI-GPx when compared to control, with a peak induction of 4.5-fold at 12 hours for TrxR1 and a peak of ~3.5-fold at 24 hours for GI-GPx, where the amount of mRNA returned to basal levels after 48h for TrxR1 but only declined slightly for GI-GPx (Figure 2.3), which imply that the mRNA turnover rate for both genes is different. The isothiocyanates tested were also showed to promote a significant induction of TrxR1 and GI-GPx expression dose-dependently with an increase of ~6- and ~3.5-fold respectively for the highest concentration (Figure 2.4), observing always a tendency of sulforaphane to promote a greater mRNA induction for both genes in comparison to iberin. Similar levels of TrxR1 induction have been observed after treating Caco-2 cells for 24 h with 5 μM of iberin (Jakubikova et al. 2006).

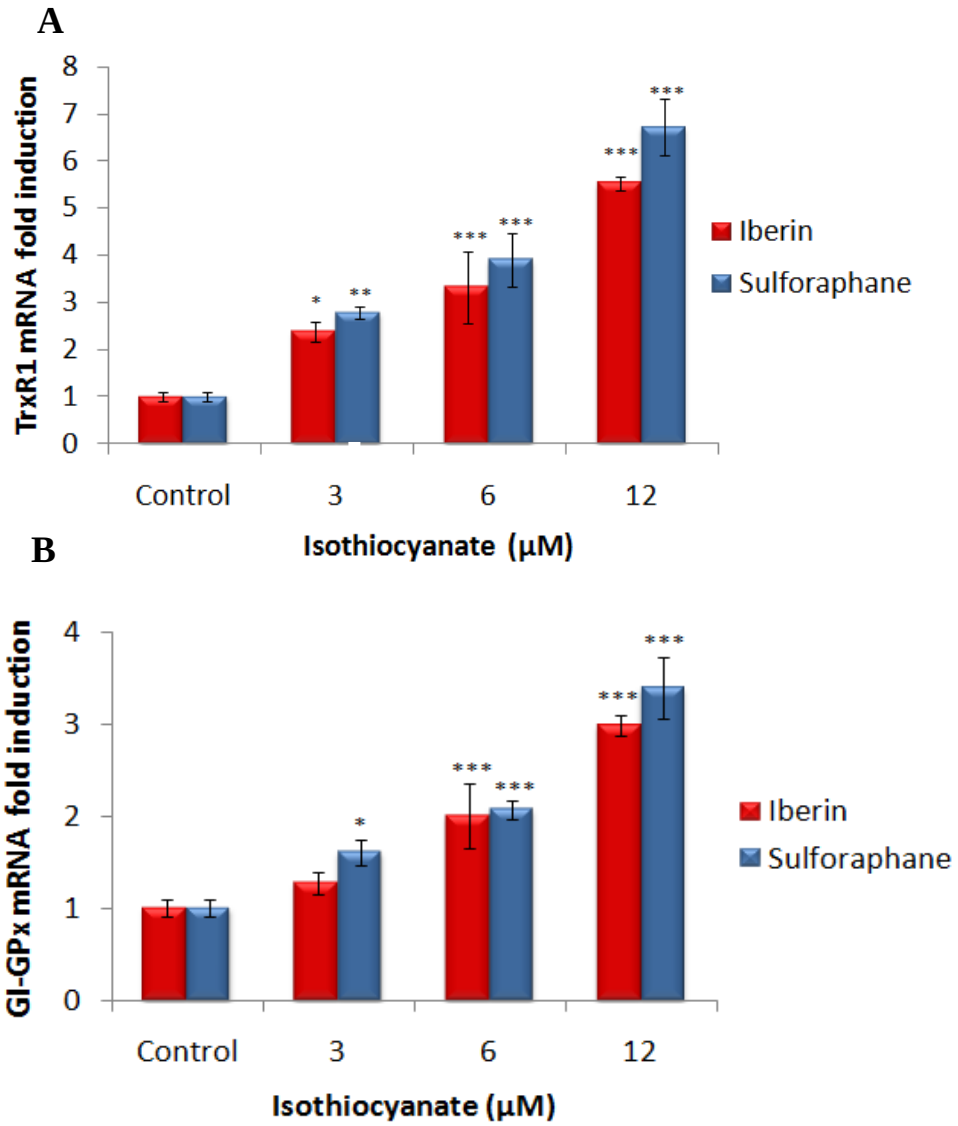


Figure 2.4 Dose dependent effects of isothiocyanates on mRNA expression. Caco-2 cells were exposed to DMSO (control) or increasing concentrations of isothiocyanates for A) 12 h for TrxR1 and B) 48 h for GI-GPx. Results are mean ± SD of triplicate samples normalized against 18S rRNA. Significant differences from control are indicated (**P*<0.05; ***P*<0.01; ****P*<0.001)

Following these results, an experiment was conducted to assess the effect on levels of TrxR1 and GI-GPx mRNA expression of selenite and SeMSC, first individually and then in combination with sulforaphane and/or iberin (Figure 2.5 and 2.6).

The amount of TrxR1 or GI-GPx mRNA was not increased by either form of selenium within 48 h. However, the combination of iberin and selenite increased *TrxR1* expression by 41% after 8 h as compared with iberin alone (p<0.05), without a significant increase when iberin and SeMSC were added together. This level of induction of 4-fold was similar to that produced by SFN alone after 24 h or SFN+Selenium at 8 or 24 h. After 24 h an increase of ~29% was observed when iberin was added with selenite or SeMSC (p<0.05).

Although a significant synergistic induction was not observed when sulforaphane was added with either form of selenium, a comparable trend was seen, especially at 8 h. TrxR1 mRNA expression tended to decrease after 48 h with only SFN plus SeMSC remaining 2-fold significantly increased compared with control and also still greater than observed in the presence of only sulforaphane or iberin (Figure 2.5). The maximum increase for this gene was detected within the sulforaphane group (SFN+SeMSC) at 24 h with a peak of 4.3-fold. Moreover, the level of induction obtained in this group remained significantly different from the iberin group (P<0.01).

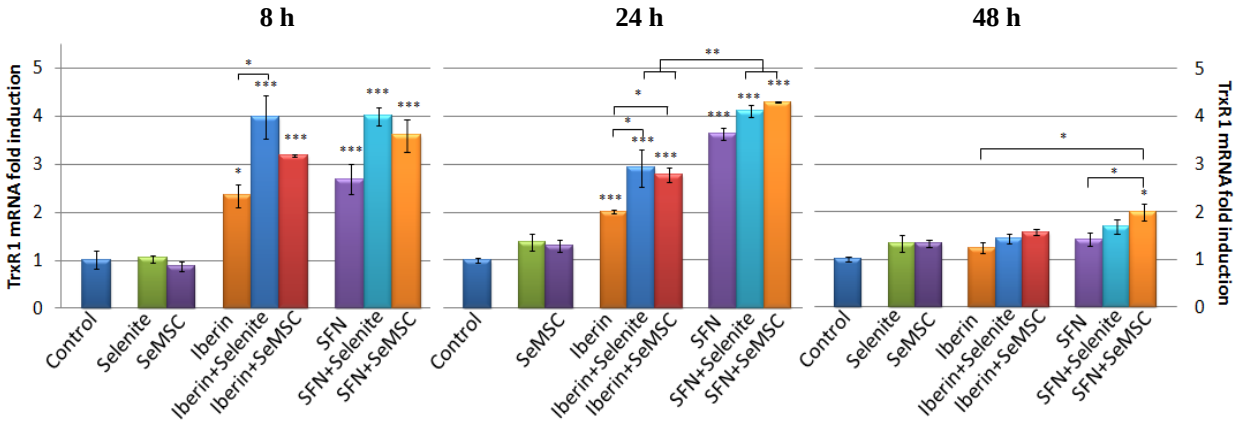


Figure 2.5 Effect of ITCs and/or Se supplementation on TrxR1 mRNA. Caco-2 cells were exposed to DMSO (control), 6 µM of ITCs and/or 200nM of selenium and were incubated for 8, 24 and 48 h to evaluate gene expression. Results are mean ± SD of triplicate samples normalized against 18S. Significant differences from control and between treatments are indicated (*P<0.05; **P<0.01; ***P<0.001). Although the majority of bars have some degree of significance the more relevant only are indicated on the graph.

In relation to the changes observed in GI-GPx mRNA, although SFN plus SeMSC had a significant effect relative to the iberin group after 8 and 48 h, the addition of selenium had no significant effect on mRNA expression when compared to ITCs alone. GI-GPx mRNA was induced 2-fold with 8 h treatment within the iberin group and was increased to 3-fold at 24 h but declined thereafter, showing a maximum 4.2-fold induction at 24 h with the isothiocyanate sulforaphane, which was significantly different from iberin (P<0.01) (Figure 2.6).

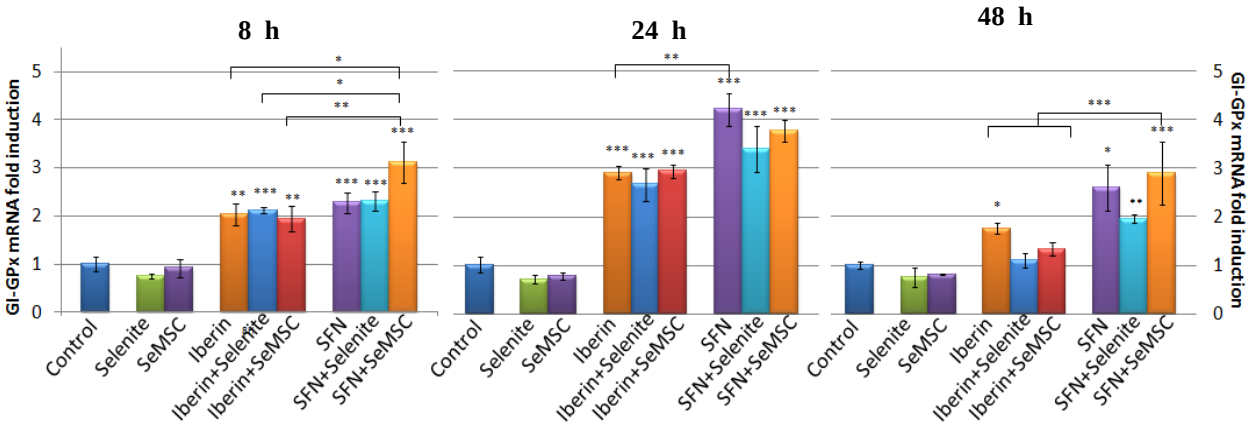


Figure 2.6 Effect of ITCs and/or Se supplementation on GI-GPx mRNA. Caco-2 cells were exposed to DMSO (control), 6 µM of ITCs and/or 200nM of selenium and were incubated for 8, 24 and 48 h to evaluate gene expression. Results are mean ± SD of triplicate samples normalized against 18S rRNA. Significant differences from control and between treatments are indicated (*P<0.05; **P<0.01; ***P<0.001). Although the majority of bars have some degree of significance the more relevant only are indicated on the graph.

To examine whether the mRNA induction of these two selenoproteins is translated into functional protein, Caco-2 cells were treated for 48 h with different concentrations of ITCs and/or selenium (Figure 2.7 A, B and 2.8 A, B) and for 8, 24 and 48 h to carry out a time course experiment (Figure 2.7C and 2.8 C), then proteins were extracted and quantified by Western Blotting as specified in material and methods.

As mRNA is ultimately translated into protein, one might assume that there should be a reasonable correlation between the level of mRNA and that of protein. However, ITC treatment is not seen to up-regulate the expression of the studied selenoproteins at the protein level as observed at the mRNA level.

On the other hand, ITC+Se TrxR1 protein induction pattern (Figure 2.7) was seen to correlate comparatively well with the mRNA level observed in Figure 2.5 in contrast to the GI-GPx protein pattern observed in Figure 2.8 which

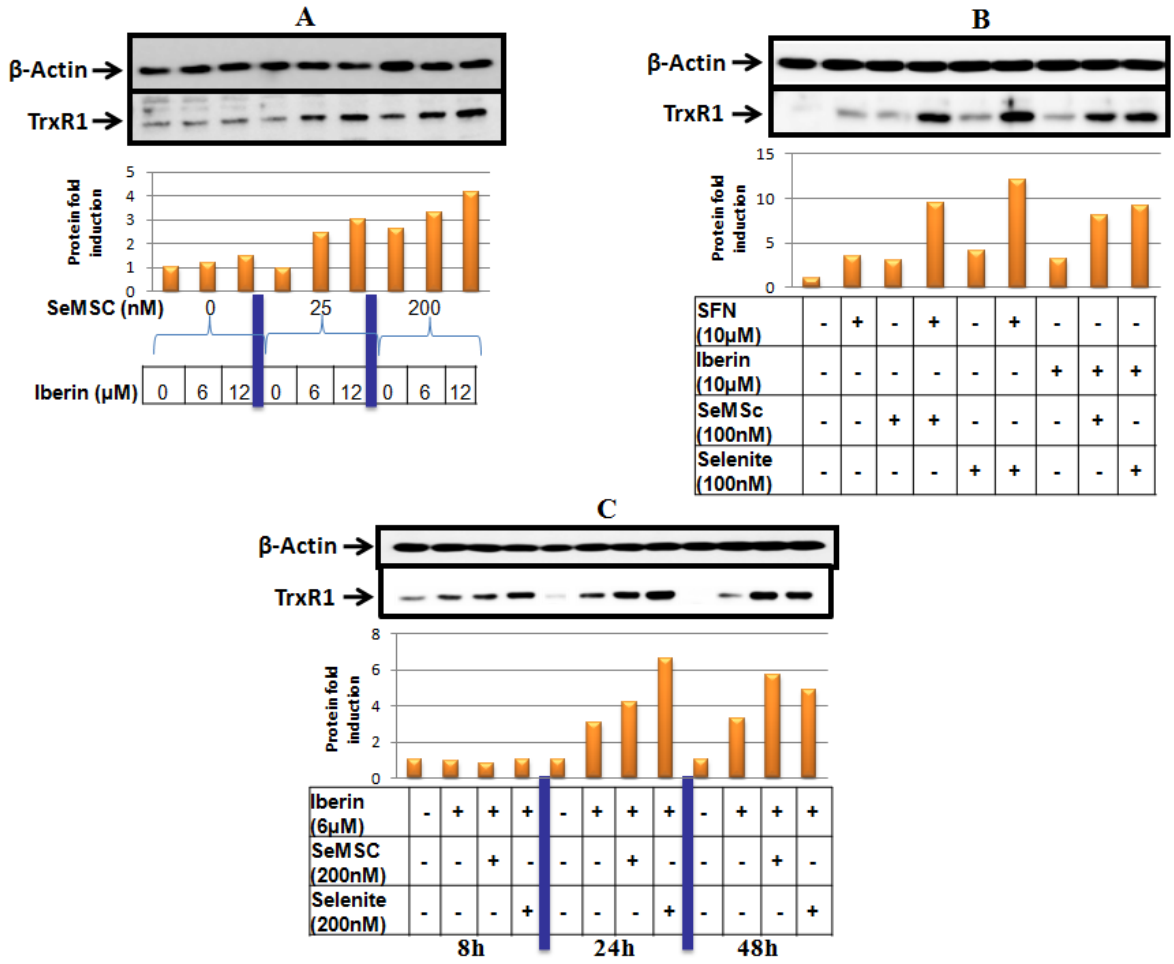


Figure 2.7 Differential levels of TrxR1 protein expression in Caco-2 cells following supplementation of ITCs and/or Se for 48 h (**A** and **B**) and for 8, 24, and 48 h (**C**). Whole-cell lysates were harvested and equal amounts of proteins (60 μg) were resolved by SDS/10% PAGE for Western blot analysis. The translated TrxR1 product appeared as a band of approximately 55 kDa and densitometric analysis of these bands were normalized to β-actin signal (42 kDa) and was converted into fold of induction relative to control=1

differed clearly from that obtained at the mRNA level, where the former appears to be visibly induced by both treatment with selenium alone and augmented by co-treatment with ITCs+Selenium in a concentration- and time-dependent manner. These results are consistent with those by Brigelius-Flohé

and co-workers who found that transcription of the GI-GPx gene was not affected by selenium supplementation in Caco-2 and HepG2 cells, but produced an increase at the protein level when cells were supplemented with 50 nM of sodium selenite (Wingler et al. 1999).

Moreover, results in Figure 2.6 showed a trend toward reduced GI-GPx mRNA when cells were supplemented with selenite or SeMSC, which also correlates with Wingler and colleague's finding where they showed higher GI-GPx mRNA expression during selenium deficiency than under selenium treatment. In this respect, according to data shown as Figures 2.7- and 2.8-A, selenium

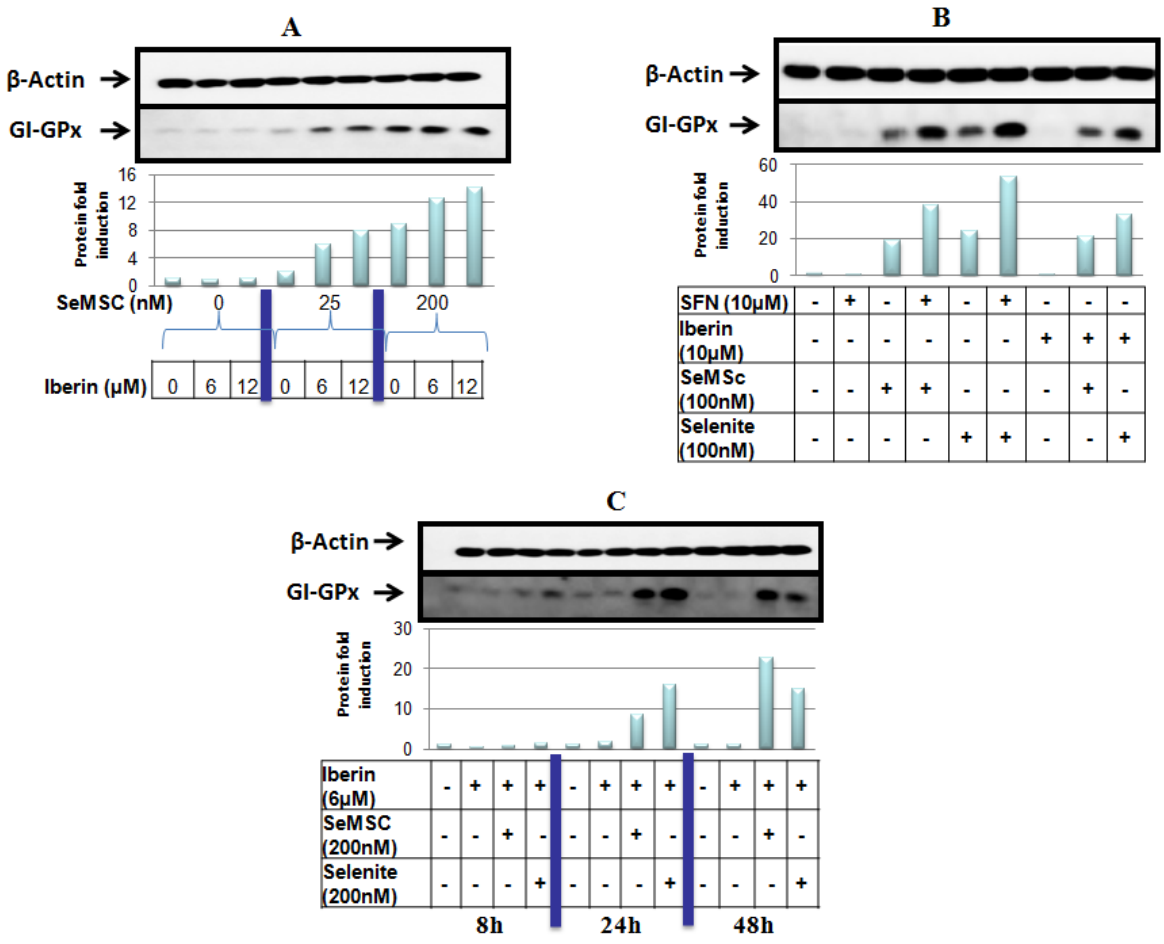


Figure 2.8 Differential levels of GI-GPx protein expression in Caco-2 cells following supplementation of ITCs and/or Se for 48 h (**A** and **B**) and for 8, 24, and 48 h (**C**). Whole-cell lysates were harvested and equal amounts of proteins (60 μg) were resolved by SDS/10% PAGE for Western blot analysis. The translated GI-GPx product appeared as a band of approximately 22 kDa and densitometric analysis of these bands were normalized to β-actin signal (42 kDa) and results were converted into fold of induction relative to control=1

was shown to be the limiting nutrient, determining the overall synergy at the protein level when cells were supplemented with ITCs and selenium dose-dependently. This observation is in contrast to what was observed at the mRNA level where the level of expression appeared to be more responsive to ITCs than selenium. The Western blotting data indicated a maximal protein induction of 4-fold and 14-fold for TrxR1 and GI-GPx respectively when cells were co-treated with 12 µM of iberin and 200 nM of SeMSC.

Immunoblot analyses of protein extracts from Caco-2 cells after treatment with both forms of ITCs and/or Se for 48 h (Figure 2.7- and 2.8-B) showed a similar pattern where sulforaphane with either form of selenium induced more protein than the co-treated iberin group. Furthermore, the SFN+selenite treatment had a tendency to be more effective in increasing protein expression than SFN+SeMSC for both selenoproteins, with a maximum level of induction of 12-fold for TrxR1 and a dramatic 53-fold induction for GI-GPx when compared to control, which represented an absolute increase of 21% and 32% respectively in comparison to the SFN+SeMSC group. Also, although iberin+selenite led to a maximum peak of protein at 24 h (~7-fold) (Figure 2.7C) the same treatment decreased protein synthesis to 5-fold after 48 h, whereas the iberin+SeMSC treatment resulted in a continued increase from 4- to 6-fold. Regarding GI-GPx (Figure 2.8-C), it was observed that Caco-2 cells supplemented with iberin+SeMSC can sustain a more prolonged increase in protein expression than iberin+selenite, increasing from 8-fold (24 h) to 22-fold (48 h) for the former.

Based on the results obtained with the different forms of selenium used, it could be inferred that the species of selenium affects the synthesis rate of selenoproteins (Figure 2.7- and 2.8-B) with a higher synthesis rate observed for the ITCs+selenite treatment compared to the ITCs+SeMSC treatment. Indeed, it has been observed that Se from high-selenium broccoli (mainly Se-methylselenocysteine) does not accumulate in tissue or increase GPx1enzyme activity to the same extent as selenite or selenomethionine, presumably because

the SeMSC is metabolised directly to methyl selenol and enters the excretory pathway (Finley 2003). In contrast, another research group reported that SeMSC induced GPx1 to a similar extent to selenite in a selenium deficient Caco-2 cell model (Zeng et al. 2008). In this study, when a time course experiment was carried out, SeMSC was able to maintain a more sustained protein up-regulation than selenite after 48 h for both genes (Figure 2.7- and 2.8-C), which represents an unknown advantage over selenite that will have to be further investigated and validated. Furthermore, Se from high-selenium broccoli has been found to be more effective than selenate or selenite in inhibiting colon cancer, which may be directly related to generation of its metabolite methyl selenol (Finley and Davis 2001), but might also be due to modulation of specific selenoproteins (such as TrxR1 and GI-GPx) differently over the long term as seen in our study in Caco-2 cells.

Sulforaphane was shown to exhibit greater up-regulation of TrxR1 or GI-GPx when in combination with either form of selenium than iberin plus selenium. Most studies examining the structure-activity relationships between different isothiocyanates against tumour growth have been conducted in arylalkyl isothiocyanates, which contain a phenyl group in their structure, such as phenethyl-ITC (PEITC) (Table 1.1). They have shown that the length of the phenylalkyl moiety of isothiocyanates affected the inhibitory potency. The inhibition of lung tumorigenesis increased as the alkyl chain was increased from 1 to 6 methylene groups; the synthetic phenylhexyl isothiocyanate (PHITC; [C6]) was approximately 50−100 times more potent than PEITC [C2], and this pattern was equally seen for other ITCs: PPeITC [C5] > PBITC [C4] > PPITC [C3] > PEITC [C2] > BITC [C1] (Morse et al. 1991; Jiao et al. 1994). However, when it comes to study the influence of different ITCs on the induction of antioxidant enzymes the level of agreement among researchers appears to be different. For instance, Munday and colleagues have stated that among isothiocyanates the length of the carbon chain appears to be of little importance, since no significant differences in inductive activity were recorded between iberverin and erucin or between sulforaphane and iberin after

analysing the induction of NQO1 and GST in a variety of rat tissues (Munday and Munday 2004). In contrast, structure-activity studies conducted by Zhang et al have examined the effects of altering the number of methylene groups in sulforaphane in NQO1 in murine hepatoma cells and have found that all of these analogues were considerably less potent than the parent sulforaphane (Zhang et al. 1992).

The synergy observed in this research has been reported previously in human HepG2 cells supplemented with sulforaphane and selenium (Zhang et al. 2003); after adding each compound separately sulforaphane caused an increase in TrxR1 expression, contrasting with selenium, which had no effects. However, after adding selenite (120 nM) plus sulforaphane (6 μM) in combination, a simultaneous increase in mRNA and *TrxR1* activity was observed beyond the maximum increase caused by either compound alone, corresponding to an induction of 6.5-fold (after 24 h) and 13.2-fold (after 72 h) respectively. This enhanced TrxR1 enhanced response was also seen after treating human hepatocytes HHL-5 with broccoli sprout extract (70% ITCs were SFN and 25% were iberin) and selenite (Li et al. 2008).

Similar results regarding the up-regulation of TrxR1 have been observed in another study comparing three dietary ITCs in a human breast adenocarcinoma cell line (MCF-7). Although this study found that selenite had no additional effect on sulforaphane-induced TrxR1 mRNA, co-addition of 200 nM of selenite with 12 μM of either sulforaphane, erucin or iberin after 48 h, significantly enhanced TrxR1 protein expression and its activity by 5.1-, 9.0-, 6.3-fold respectively, showing that erucin and iberin possess superior activity to that of sulforaphane (Wang et al. 2005), a difference from the results obtained in this study where sulforaphane was seen to exert a greater induction than iberin in Caco-2 cells. Likewise, the ability of sulforaphane and selenium to modify the mRNA expression of TrxR1, GPx1and GPx4 was assessed in the human endothelial cell line EAhy926, identifying that selenite alone increased significantly the expression of all the studied selenoenzymes, in contrast to

sulforaphane, which only increased TrxR1. This enzyme was also the only one that showed a significant synergistic increase when SFN+selenite were added to the media.

Key studies have identified that the mRNA levels of the selenoproteins evaluated here are transcriptionally modulated via an antioxidant responsive element (ARE), which facilitates rapid expression responses unrelated to the established Se-dependent induction of selenoproteins (Rundlof et al. 2001; Hintze et al. 2003; Banning et al. 2005). In this respect, it has been suggested that the modulation of TrxR1 activity by SFN and selenium occurs independently. The former works transcriptionally through the ARE located in the promoter region of the gene. This process involves disruption of the Keap1-Nrf2 complex by inducer SFN leading to Nrf2 migration to the nucleus where it binds to the ARE region of TrxR1 or GI-GPx in the promoter region of the gene. On the other hand, selenium supply is thought to regulate TrxR1 expression by post-transcriptional mechanisms that include the provision of an adequate supply of selenocysteine for incorporation into the TrxR1 protein, delaying its degradation (Gallegos et al. 1997; Hintze et al. 2003; Zhang et al. 2003). These previously described findings are in agreement with our results obtained for TrxR1, but also fit with results observed for GI-GPx were selenium was the limiting nutrient at the protein level (Figure 2.7- and 2.8-A), indicating a similar mechanism of regulation for both selenoenzymes.

Since GI-GPx and TrxR1 are selenoproteins, their expression and activity are regulated by selenium, where adequate selenium supply is critical for synthesis of selenoproteins through the selenocysteine insertion mechanism. However, as observed in this research, the interrelationships between selenium supplementation and selenoprotein expression are complex and will depend upon the type of selenium source, the concentration of selenium and, according to other studies; it will also depend upon the cell-type under consideration. On the other hand, as confirmed here, this regulation occurs at different levels for different selenoenzymes, which means that selenoproteins are regulated

individually through changes in their mRNA and protein levels, as seen for GI-GPx mRNA, which was not seen to be influenced consistently when selenium was combined with ITCs during the different times evaluated, a difference from TrxR1 mRNA, which was seen to be more responsive to selenium. This variation among selenoproteins is believed to be as a result of differences in the 3´UTR sequence (where the selenocysteine insertion sequence SECIS is present), which might be responsible for differences in relative transcription rates and mRNA stability, probably reflecting differences in ability to form a complex with Sec-tRNA and the proteins forming the SECIS-binding complex (Hesketh and Villette 2002; Reilly 2006). For comparison with the current data, some studies have reported that GI-GPx is not influenced during selenium deficiency, whereas GPx1 decreases and GPx4 mRNA levels remain unaffected (Wingler et al. 1999), with TrxR1 being also reported to decrease under conditions of limiting selenium supply (Gallegos et al. 1997; Crosley et al. 2007). This corroborates different levels of regulation and might imply a 'prioritisation' of available Se so that synthesis of some selenoproteins is maintained more than others. From this phenomenon, called hierarchy of selenoproteins, it has been concluded that selenoproteins ranking high in the hierarchy, i.e. remaining stable under selenium restriction (such as GI-GPx), might have more essential functions than those ranking low (Brigelius-Flohé and Banning 2009).

2.2.3 Regulation of Nrf2-mediated Antioxidant Enzyme Induction

2.2.3.1 Nrf2 Transcriptional and Translational Expression Pattern in ITCs and/or Selenium Treated Caco-2 Cells

The proposed molecular mechanisms through which ITCs activate phase II enzymes and the selenoproteins investigated here involve the Keap1-Nrf2-

antioxidant response element (ARE) (Hintze et al. 2003; Banning et al. 2005). In the absence of ARE inducers such as ITCs, Nrf2 protein is found primarily in the cytoplasm of cells where its concentration is maintained at low levels. An important mechanism controlling the increase of Nrf2 protein level is through Keap1, an inhibitor of Nrf2, known to bind Nrf2 and hold it in the cytoplasm, where Keap1 recruits an E3 ubiquitin ligase, resulting in Nrf2 ubiquitination and therefore degradation by the proteosome (Eggler et al. 2008). Also, an adequate selenium status has for a long time been considered to prevent the development of various forms of cancer. Recently it was found that selenium deficiency increased the level of reactive oxygen species (ROS), activating the Nrf2–ARE pathway in the livers of male *Nrf2*$^{+/+}$ mice promoting as a result a remarkable over-expression of multiple antioxidant proteins (Burk et al. 2008). On the other hand, *Nrf2*-deficient mice have been shown to display reduced expression levels of phase 2 enzymes and an increased susceptibility to carcinogenesis (Ramos-Gomez et al. 2001).

However, the underlying mechanisms by which ITCs+Se up-regulate further the gene expression of intracellular antioxidants need to be addressed. In order to confirm if the mechanisms involved in the induction of antioxidant enzymes when Caco-2 cells are co-treated with ITCs and selenium is also Nrf2 dependent, mRNA and protein levels in combination with knockdown experiments were studied in Caco-2 cells treated with iberin and/or SeMSC to decipher the implication of this important nuclear factor in the studied synergy of TxR1 and GI-GPx.

The results showed no elevation of Nrf2 mRNA when cells were treated in a time-dependent manner with ITCs and/or selenium after 8 h (Figure 2.9). Significant subtle increased of 1.5- and 1.3-fold were observed at 24 and 48 h when cells were treated with SFN or SFN+SeMSC respectively.

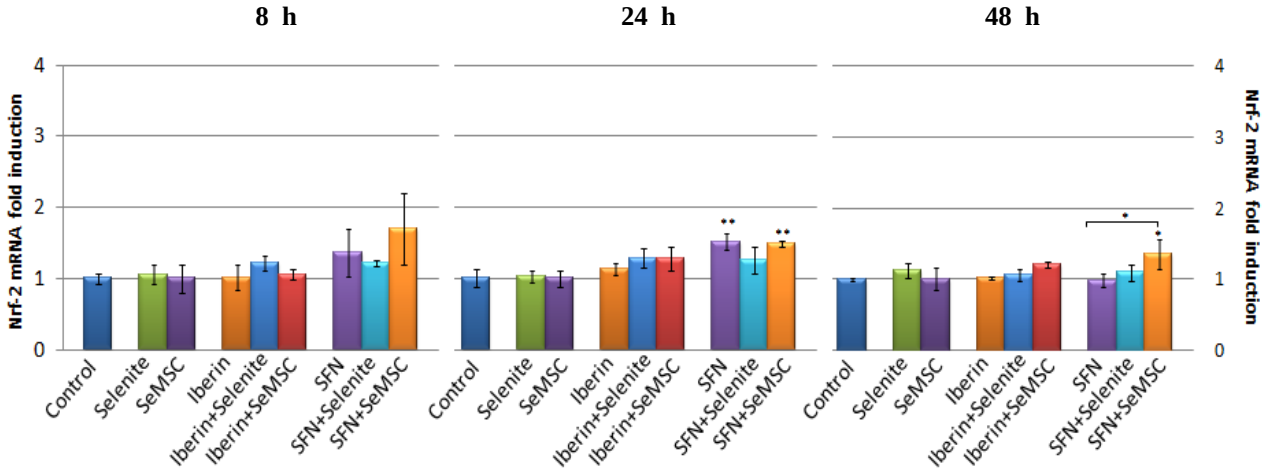


Figure 2.9 Effect of ITCs and/or Se supplementation on *Nrf2* mRNA. Caco-2 cells were exposed to DMSO (control), 6 µM of ITCs and/or 200nM of selenium and were incubated for 8, 24 and 48 h to evaluate gene expression. Results are mean ± SD of triplicate samples normalized against 18S. Significant differences from control and between treatments are indicated (**P*<0.05; ***P*<0.01).

On the other hand, nuclear proteins revealed a different pattern, showing a 3-fold Nrf2 induction after exposing Caco-2 cells for 8 hours to the isothiocyanates SFN or iberin. The concomitant decreased levels of Nrf2 observed in the cytosolic fraction (Figure 2.10) when cells are treated with these food constituents reflect the active translocation of Nrf2 from the cytosol to the nucleus due to the treatment of cells with an ARE inducer, such as isothiocyanate, that inhibit ubiquitination of Nrf2, leading to its nuclear accumulation and activation of cytoprotective enzymes. However, it was noticed that the treatment corresponding to ITCs+Se did not increase further the expression of Nrf2. This founding may indicate that the synergistic induction of TrxR1 at the mRNA and protein level and GI-GPx (observed mainly at the protein level) is not Nrf2 dependent. Previous studies have suggested that although Se does not induce TrxR1 mRNA, Se can delay the degradation of sulforaphane induced TrxR1 mRNA (Zhang et al. 2003). In this respect, selenoprotein mRNAs are potential targets for degradation via nonsense-mediated decay (a quality-control mechanism that selectively degrades mRNAs harbouring premature termination (nonsense) codons (Chang et al. 2007)) due to the presence of in-frame UGA codons that can be decoded as either selenocysteine (Sec) or termination codons. When UGA decoding is

inefficient, as occurs when selenium is limiting, termination occurs at these positions (Squires et al. 2007) favouring mRNA degradation, which means that Sec-tRNA^{sec} concentration (see section 1.5.1) control selenoprotein expression not only by limiting translation but also by modulating mRNA stability (Hatfield 2001). This mechanism might imply that the observed induction of TrxR1 and GI-GPx in Caco-2 cells after co-addition of ITC+Se potentially responds to a reduced mRNA degradation rate rather than over-stimulation of the Nrf2-ARE signaling pathway (induced by ITC) when selenium is added.

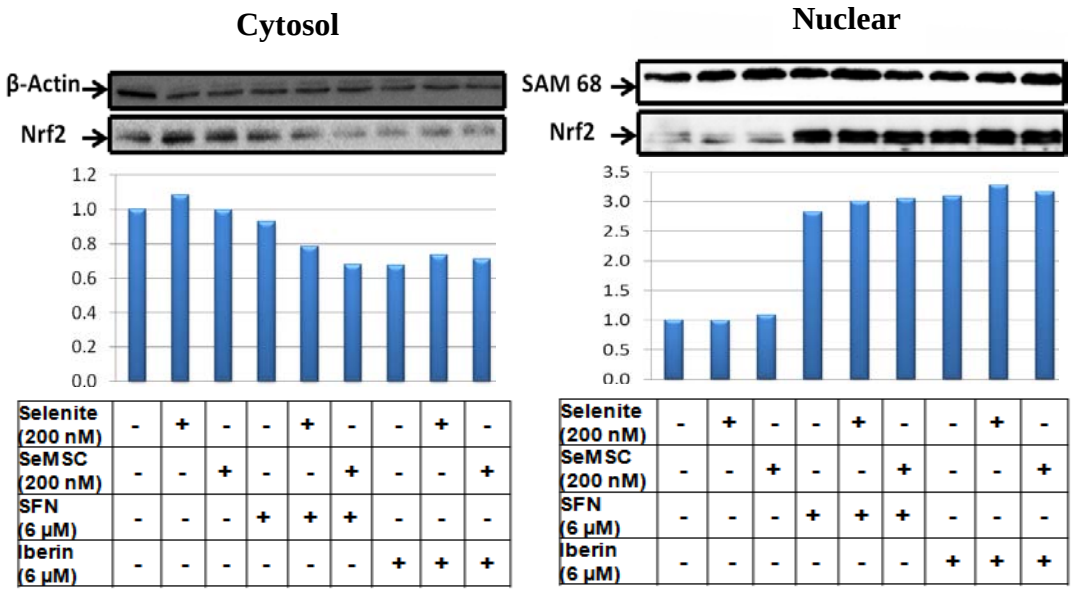


Figure 2.10 Representative images of Nrf2 immunoblots with cytosolic and nuclear fractions derived from Caco-2 cells exposed for 8h with DMSO (control), SFN, SFN+Selenium, iberin or iberin+Selenium. Cellular fractions (30 µg) were resolved by SDS/10% PAGE. Cytosolic and nuclear bands were detected using anti-Nrf2 (C-20) and anti-Nrf2 (H-300) antibodies respectively and bands were seen at 57- (cytosol) and ~100-kDa (nuclear). Densitometric analysis of cytosolic and nuclear bands were normalized to β-actin (42 kDa) and Sam 68 (68 kDa) signals respectively and results were converted into fold of induction relative to control=1.

It should be noted that the theoretical molecular weight of Nrf2 is about 67KDa for humans, mice, and rats. But, it has been noted that the apparent molecular weight of human or murine Nrf2 in SDSPAGE ranges from 57 KDa to 110 KDa (Li et al. 2005). The occurrence of the higher molecular mass of Nrf2 in Western blot analysis has been suggested to represent a Nrf2-actin complex

regulated by the PI3-kinase signalling pathway and the result of sequential phosphorylation by protein kinase CK2 after the Nrf2 is transported to the nucleus (Kang et al. 2002; Pi et al. 2007).

A discrepancy between mRNA and protein levels for Nrf2 similar to the observation made here has been observed previously in freshly isolated neonatal rat cardiomyocytes cells treated with 100 µM H_2O_2 for 10 min, at which time the media was changed and cells were harvested at different times for a total period of 4 h. Results revealed that semiquantitative or RT-PCR did not show an increase of Nrf2 mRNA level, but that Western blot analysis showed a rapid accumulation of Nrf2 protein within 10 min, with a peak of 2-fold at 1 to 2 h after H_2O_2 treatment (Purdom-Dickinson et al. 2007). Also, despite Zhang and colleagues reporting an up-regulation in the Nrf2 mRNA and protein levels in Caco-2 cells treated with epigallocatechin-3-gallate (EGCG), their reported increase in Nrf2 protein corresponded to subtle changes of ~1.3- and 1.4-fold respectively compared to control cells (Zhang et al. 2009), in contrast to the 3-fold protein increase after iberin+SeMSC treatment observed in this study (Figure 2.10).

2.2.3.2 *Nrf2* siRNA Interference Assay

To further confirm that the molecular mechanism of action through which ITCs modulate TrxR1 (Figure 2.11 A) and GI-GPx (Figure 2.11 B) is Nrf2 dependent, we knocked down the expression of Nrf2 in Caco-2 cells and simultaneously treated them with ITCs and or selenium . The siRNA significantly (P<0.05) reduced the expression of Nrf2 by 46% (Figure 2.11 C). Although the Nrf2 knockdown efficiency obtained was low, the average reduction in TrxR1 and GI-GPx induction after suppressing Nrf2 equalled that observed for the knockdown efficiency (~44.8%), except for the treatments iberin and iberin+selenite within the TrxR1 and GI-GPx groups respectively, where the reduction obtained was only 35 and 15%. These results demonstrate

the role of Nrf2 to induce the expression of TrxR1 or GI-GPx in colon cancer cells in response to ITCs alone as explained in the previous section.

Many studies have been carried out to understand the mechanism of action through which certain chemoprotective agents, among them isothiocyanates, promote the translocation of Nrf2 into the nucleus (Jakubikova et al. 2006; Jeong et al. 2006) and it has been proposed that sulforaphane prevents the phosphorylation of Nrf2 by inhibiting p38 MAPK isoforms in HepG2 cells, resulting in a reduced interaction between Keap1 and Nrf2 and subsequent *Nrf2* activation (Keum et al. 2006). Additional mechanistic studies focussing on the Nrf-2–ARE signalling pathway have shown JNK1 as a n upstream inducer of Nrf-2 to activate ARE-containing genes by phenethyl isothiocyanate in HeLa cells (Keum et al. 2003).

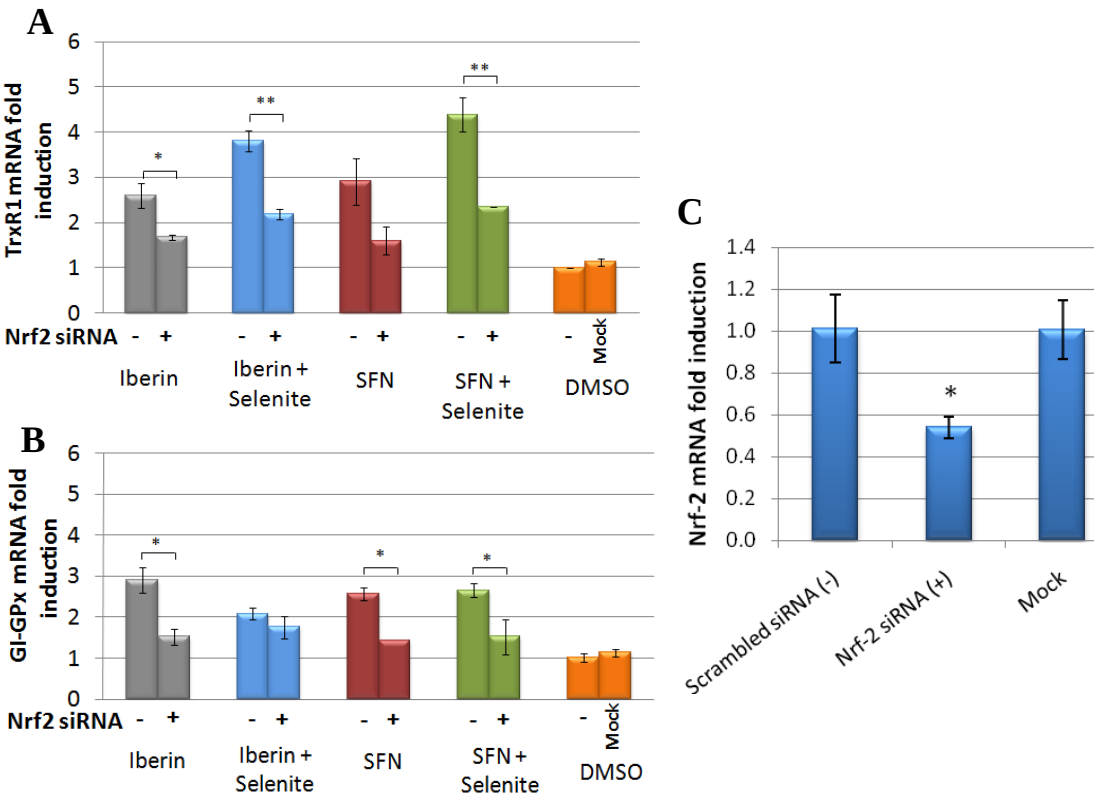


Figure 2.11 Effect of ITCs and/or selenite treatment on A) TrxR1 and B) GI-GPx expression in *Nrf2* suppressed Caco-2 cells. Caco-2 cells were exposed to either 30 nM of silencer negative scrambled siRNA (-) or 30 nM of *Nrf2* siRNA (+) with the carrier Lipofectamine 2000 for 24 h before adding vehicle DMSO, ITCs (6 µM) and/or Selenite (200 nM) for 12 h. Mock transfected cells received only Lipofectamine 2000. C) *Nrf2* knockdown efficiency. Results are mean ± SD of triplicate samples normalized against 18S rRNA. Results were converted into fold of induction relative to control (DMSO treated cells containing scramble siRNA (-) depicted in orange). Significant knockdown (+) from basal levels (-) are indicated (**P*<0.05; ***P*<0.01).

2.2.4 Effects of Hydrogen Peroxide on Caco-2 Cells Pre-treated with Sulforaphane and/or Selenite.

2.2.4.1 Cytotoxicity of Hydrogen Peroxide

The cytotoxic effect of H_2O_2 was evaluated by treating Caco-2 cells with increasing concentrations of H_2O_2 from 200 to 800 µM. The results indicated that doses above 200 µM of H_2O_2 evoked a remarkable statistically significant decrease in cell viability dose-dependently compared to control (Figure 2.12A). In addition, when Caco-2 cells were pre-incubated with selenite (50 nM) for 24 h followed by exposure of increasing concentrations of H_2O_2 (ranging from 600-1200 µM) for an additional 24 h, although a dose-dependent reduction in cell viability was observed, pre-treatment of the cells with selenite reduced the toxicity of hydrogen peroxide compared to the untreated group. For instance, when cells were treated with 600 and 800 µM of H_2O_2 (Figure 2.12 B) the cell protection increased by 34 and 41% respectively compared to the untreated selenium group (Figure 2.12 A). Also, the IC_{50} value for the untreated and pre-treated selenite group corresponds to ~500 and 950 µM respectively which indicates a decrease in cell sensitivity as a result of the increased cellular protection offered by selenite.

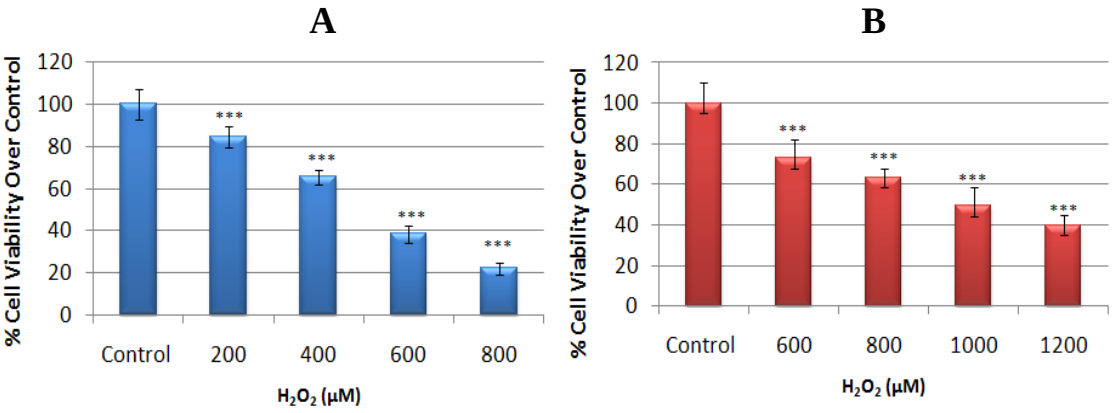


Figure 2.12 The cytotoxic effect of H_2O_2 on cells A) untreated or B) pre-treated with selenite. Caco-2 cells were grown for 48 h, and then treated for 24 h with vehicle (A) or with selenite (50 nM) (B) followed by treatment with increasing concentration of H_2O_2 for 24 h as shown above. Cell cytotoxicity was evaluated by WST-1 assay. Results are mean ± SD of six replicates (***P<0.001).

2.2.4.2 ITCs and/or Se-mediated Cytoprotection in Caco-2 Cells

In this study, co-addition of isothiocyanates and selenium resulted in a synergistic induction of *TrxR1* and *GI-GPx* expression in a time- and dose-dependent manner at the transcriptional and translational level. Therefore, we sought to identify whether this increase was relevant for cell protection against

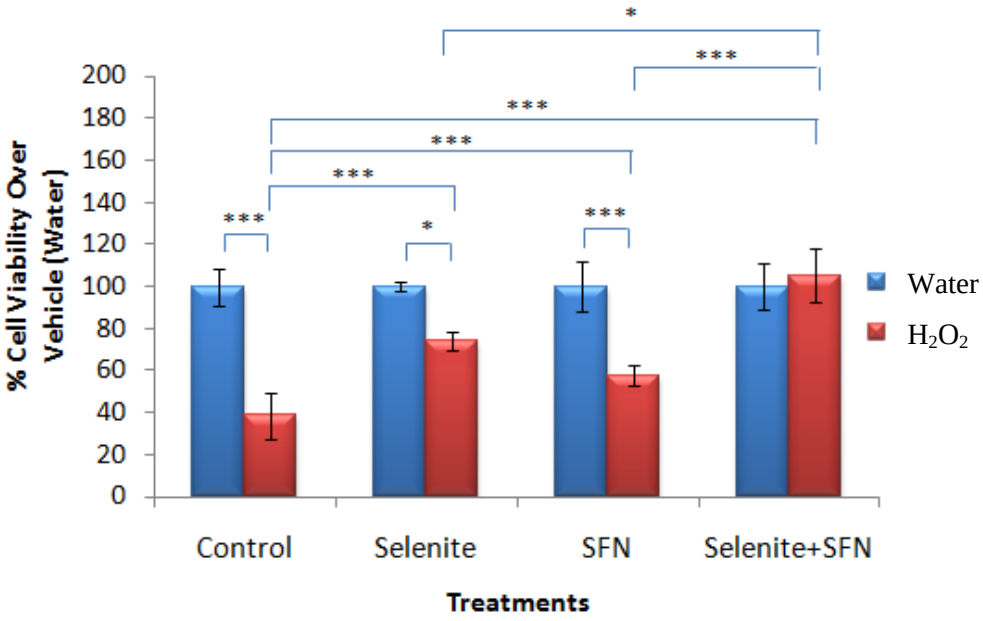


Figure 2.13 Effect of ITCs and/or selenium on cell viability in Caco-2 cells treated with hydrogen peroxide. Caco-2 cells were pre-incubated for 24 h with DMSO (control), SFN (6 µM) and/or selenite (50 nM) before exposure to H_2O_2 (500 µM) in serum-free medium for 24 h. Cell cytotoxicity was measured by WST-1 assay. SFN and/or selenite-mediated cytoprotection are shown in red bars as a percentage of the H_2O_2 untreated group (blue bars). Results are mean ±SD of six replicates. Significant differences from control and between treatments are indicated *P<0.05; ***P<0.001

an oxidative stress insult after pre-treating Caco-2 cells with sulforaphane (6 µM) and/or selenite (200 nM) for 24 h followed by treatment with 500 µM H_2O_2. These ITC and/or Se forms were selected as they were shown to be the ones offering greater induction of TrxR1 and GI-GPx expression.

The results showed that in the control group H_2O_2 decreased the cell viability of Caco-2 cells by 60%. However, pre-treatment of the cells for 24 h with selenite and SFN significantly enhanced cell protection by 35 and 20%

respectively compared to the control group without pre-incubation with the studied dietary compounds (Figure 2.13). Likewise, others have observed a similar level of protection when human hepatoma HepG2 were pre-treated with SeMSC for 2 or 20 h prior to being subjected to t-BOOH, a different pro-oxidant that can decompose to peroxy radicals and generate lipid peroxides and ROS (Cuello et al. 2007).

Remarkably, co-treatment with Selenite+SFN abolished completely H_2O_2-induced cell damage, providing a synergistic protection of 60% compared to H_2O_2-treated control (Figure 2.13). This level of protection was statistically significant compared to that generated by selenite ($P<0.05$) or SFN ($P<0.001$) alone.

Although this investigation only used as an endpoint a cytotoxicity assay to measure the effect of enhancing antioxidant enzymes to protect the membrane integrity of Caco-2 cells treated with ROS, others have also looked at the genotoxic level to determine the effect of different phytochemicals on DNA strand breaks. For instance, exposure of Caco-2 and HepG2 cells to 50 µM H_2O_2 for 30 min generated significant DNA damage, but pre-incubation with 10, 50 or 200 µM of the flavonoids myrcetin, quercetin and rutin before H_2O_2 exposure significantly protected cells in a dose dependent manner against H_2O_2-induced DNA damage (Aherne and O'Brien 1999). Duthie and co-workers also pre-incubated human lymphocytes with myricetin or quercetin for 30 min or 18 hours before exposure to H_2O_2 (200 µM). Pre-treatment of the human lymphocytes with myricetin or quercetin for 30 min reduced H_2O_2-induced DNA damage. Interestingly, no protection was seen after 18 hours of pre-incubation with quercetin in the human lymphocytes (Duthie et al. 1997), emphasising the importance of conducting time- and dose- dependent experiments to identify relevant points of protection within the studied system.

2.2.5 Role of TrxR1 and GI-GPx in the Synergism between ITC and Se-mediated Cytoprotection in Caco-2 Cells

2.2.5.1 *TrxR1/GI-GPx* siRNA Optimisation Assay

As mentioned in section 2.1.2.6.1 the amount of siRNA and the ratio of HiPerFect transfection reagent to siRNA were optimised first (data not shown). After this, the amount of siRNA/Hiperfect chosen was used to optimise the single and double knockdown prior to measuring the cell viability effects of *TrxR1* and/or *GI-GPx* knockdown in Caco-2 cells (section 2.2.5.2). Figure 2.14 shows that the knockdown efficiency corresponds to about 80% for both single and double gene knockdown. The mock-transfected sample (cells treated with the transfection reagent HiPerFect without addition of siRNA) showed the absence of nonspecific effects.

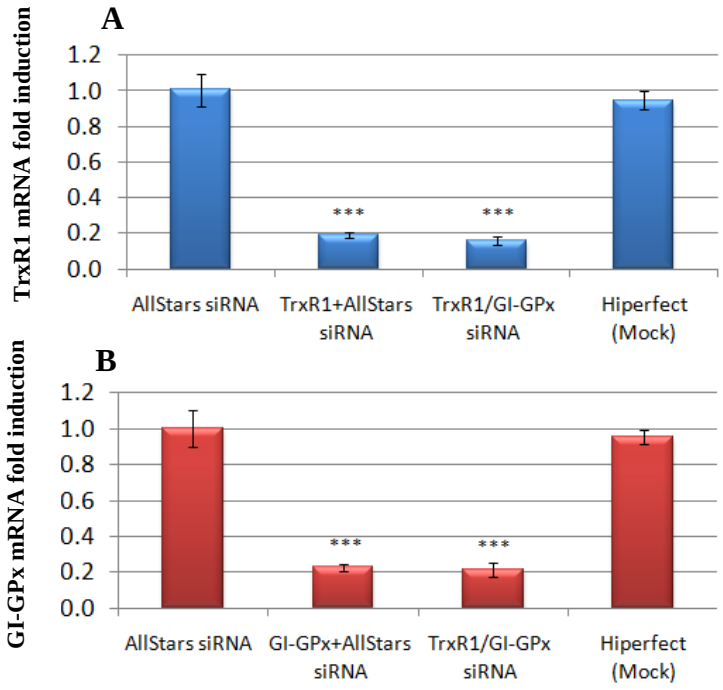


Figure 2.14 siRNA knockdown efficiency of A) TrxR1 and/or B) GI-GPx expression. Caco-2 cells were exposed to● 50 nM of AllStars negative control siRNA; ● 25 nM of *TrxR1* or *GI-GPx* siRNA+ 25 nM AllStars siRNA (single knockdown); ● 25 nM of *TrxR1* + 25 nM *GI-GPx* siRNA (double knockdown) for 24 h; Mock-transfected cells were transfected only with HiPerFect, without addition of siRNA . Then, media containing the transfection complex was replaced with culture media and left for an additional 24 h, followed by total RNA extraction. Results are mean ± SD of triplicate samples normalized against 18S rRNA. Significant differences are indicated (***P<0.001).

2.2.5.2 Effect of *TrxR1* and/or *GI-GPx* Knockdown on H_2O_2-Induced Damage in Caco-2 Cells Pre-treated with SFN+Selenite

To investigate if *TrxR1* and *GI-GPx* are the main genes by which isothiocyanates and selenium protect against hydrogen peroxide-induced cell death in Caco-2 cells, a single and double knockdown approach was used to suppress the expression of the aforementioned selenoproteins. Addition of 400 µM of H_2O_2 in control cells transfected with AllStars negative control siRNA without pre-treatment with dietary components decreased cell viability by 50% (P<0.001) (Figure 2.15). In contrast, cells transfected with AllStars negative control siRNA but pre-treated with SFN+Selenite, were shown to be 100% viable after addition of H_2O_2, confirming the level of protection observed in section 2.2.4.2. However, this level of protection was found to be affected by a single knockdown with either *TrxR1*- or *GI-GPx*-siRNA prior to a pre-incubation step with SFN+selenite and followed by an oxidative stress challenge with H_2O_2. The data showed a reduction of 15 and 25% (P>0.05) in cell viability after *TrxR1* or *GI-GPx* single knockdown respectively. Interestingly, when both selenoproteins were knocked down at the same time a ~50% reduction in cell viability (P<0.001), equivalent to that obtained in the control group without any dietary pre-treatment, was observed. This observation indicates that SFN+Selenite mediate their protective synergistic effects through both selenoproteins.

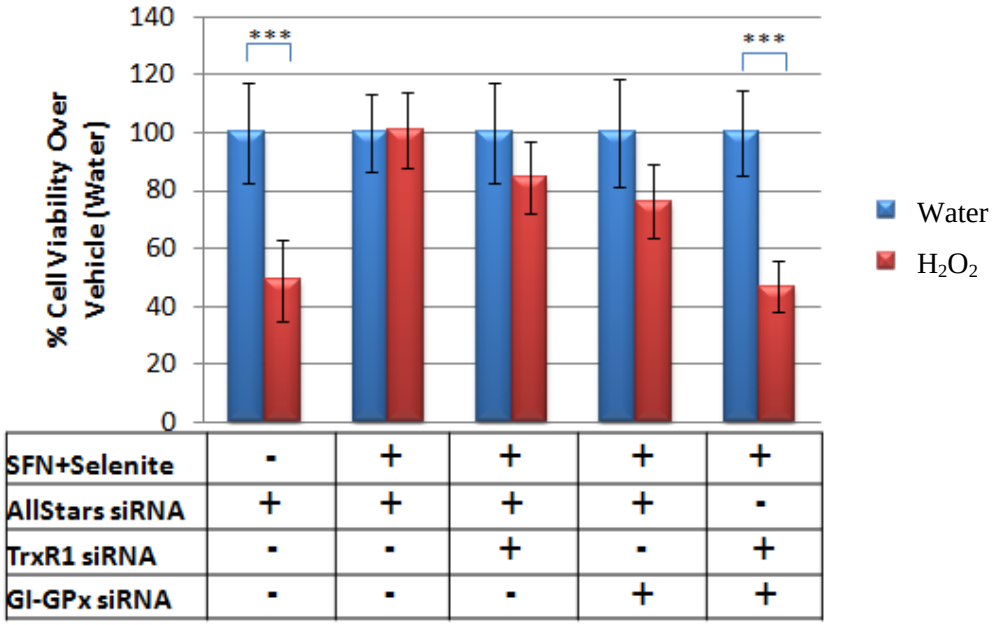


Figure 2.15 Cell viability effects of *TrxR1* and/or *GI-GPx* knockdown on Caco-2 cells, untreated or pre-treated with SFN+Se followed by incubation with vehicle (blue bar) or H_20_2 (red bars). Caco-2 cells were exposed to ● 50 nM of AllStars negative control siRNA; ● 25 nM of *TrxR1* or *GI-GPx* siRNA+ 25 nM AllStars siRNA (single knockdown); ● 25 nM of *TrxR1* + 25 nM *GI-GPx* siRNA (double knockdown) for 24 h before adding vehicle DMSO to samples containing only AllStars or SFN (6µM) + Selenite (50 nM) followed by addition of H_20_2 after 24 h. Cell viability was measured after 24 h by the WST-1 method. Results are mean ± SD of six samples. Significant differences are indicated (***P<0.001).

Zhang and colleagues carried out a similar experiment in HepG2 cells to identify the protective effect of sulforaphane and selenite against paraquat-mediated cytotoxicity, identifying that SFN at 6 µM only inhibited 20% of the lactate dehydrogenase (LDH) release, but the combination with selenium inhibited 40-60% LDH release (P<0.05) (Zhang et al. 2003). These results obtained by Zhang are in line with the data presented in this investigation, which has provided further clues to understand the mechanism involved in the protection exerted by isothiocyanates and selenium.

Taken together, accumulation of ROS in cells is thought to be a major cause of molecular injury leading to cell aging and to age-related degenerative diseases such as cancer. Therefore, the anticarcinogenic actions of cruciferous vegetables and/or selenocompounds may be attributed, in part, to their ability

to protect against oxidative stress through the activation of genes involved in the enhancement of cellular antioxidant capacity as seen in this study after addition of ITCs+Se, which potentiates TrxR1 and GI-GPx expression and reinforces the intrinsic cellular defense system to detoxify and promote the removal of potential oxidants or carcinogens stressors. However, in recent years it has also become more clear the role that *TrxR1* plays in cancer. It is well cited that *TrxR1* is highly up-regulated in human tumours and cancer cell lines (Arnér 2009) and its knockdown in a mouse cancer cell line driven by oncogenic k-ras has been seen to result in morphological changes characteristic of parental (normal) cells (Yoo et al. 2007), demonstrating that over-expression of *TrxR1* has a direct role in the carcinogenesis process. In addition, TrxR1 is the primary enzyme that reduces thioredoxin (Chapter 1; Figure 1.8), and reduced thioredoxin has been associated with increased cell growth and decreased apoptosis, conditions that promote the growth of cancerous cells (Powis et al. 1997). Consequently, this enzyme has been suggested as a target for anticancer drugs in the inhibition of the malignancy process (Smart et al. 2004).

Furthermore, the fact that *GI-GPx* is transiently increased in human colorectal adenomas compared with normal adjacent mucosa, with the highest expression in pre-cancerous polyps (adenomas) (Mörk et al. 2000) and reducing amounts in late stage of carcinogenesis, imply that this selenoprotein might also participate in the cancer development process and that its expression might depend on the developmental stage of malignant transformation (Brigelius-Flohé and Kipp 2009). This, combined with high expression in the epithelium at the crypt grounds of the small intestine and colon, where the proliferating stem cells are located, suggest a role in proliferative processes (Florian et al. 2001).

Considering that Nrf2 and its upstream signalling cascade play a vital role in ARE-driven gene expression, it has been observed that stable over-expression of Nrf2 in cancer cells promotes survival of cancer cells during treatment with

chemotherapeutic agents, resulting in enhanced resistance of cancer cells to such treatments (Wang et al. 2008). Nevertheless, it is important to mention that stimulation of the Nrf2-dependent defensive response by Nrf2 activators in normal tissue is transient because the negative regulator of Keap1 is only transitorily inhibited. However, in cancer tissues, dysregulation of Nrf2 by Keap1 due to mutation of the latter results in strong and persistent induction of Nrf2 (Ohta et al. 2008).

In line with the previous thought, it seems feasible to think that the mechanism of cell protection exhibited by this transcription factor and by selenoenzymes studied will depend upon the tissue studied (normal cells vs. tumour cells) and/or the stage of the carcinogenesis process. In normal cells, it is well known that apart from glutathione reductase, cytosolic TrxR1 is believed to be the most important enzyme for control of the cellular redox state, antioxidant defense, and redox regulation of cellular processes. In addition, the observation that TrxR1 activates the *p53* tumour suppressor in cells treated with electrophilic lipids (Moos et al. 2003; Cassidy et al. 2006) provides evidence for an important mechanism to understand how dietary selenium confers protection against cancer through the selenoprotein TrxR1. Likewise, GI-GPx has been shown to represent one of the major anti-inflammatory factor modulating oxidative stress in the gastrointestinal tract as reported in the restoration of one of the *GI-GPx* (*GPx2*) alleles in a *GPx*-1/2 KO mouse, which prevented pathological symptoms characteristic of inflammatory bowel diseases and intestinal cancer .

However, in emerging tumours, it seems that the aforementioned genes will provide signals to sustain the cancerous process. If this is true, then the results obtained from this research will be more relevant to normal cells as a cancer preventive measure before the cancer process has initiated rather than a cancer curative treatment. But, before a more robust conclusion can be drawn, further studies are warranted to determine whether up-regulation of GI-GPx, TrxR1 and Nrf2 in cancer represents a compensatory mechanism to counteract

oxidative damage or if it is part of the proliferative program which is essential for carcinogenesis.

3

Impact of Isothiocyanates and Selenium on Global and Gene-specific Methylation Patterns and Effects on DNA Methyltransferase Gene Expression in Colon Cancer Cells *in vitro*

3.0 INTRODUCTION

Promoter methylation of multiple genes has been reported in several conditions of the gastrointestinal tract including Barrett's oesophagus (Peng et al. 2008), chronic gastritis (Kang et al. 2003), ulcerative colitis (Tominaga et al. 2005) and colorectal cancer (Xu et al. 2004). Some of these changes have been suggested to be age-related (Issa et al. 1994), while others suggested an association with environmental risk exposures (Shen et al. 2002) and/or diet (Johnson and Belshaw 2008). Despite the fact that most work on the cellular effects of ITCs relate to their influence on detoxifying enzymes, more recent new data support the effect of these dietary compounds on the reactivation of epigenetically silenced genes in cancer cells, particularly through the inhibition of histone deacetylase (HDAC) activity (Dashwood and Ho 2008). These enzymes act epigenetically by deacetylating the amino-terminal tails of histones producing chromatin changes that regulate transcription and many other nuclear events (Minucci and Pelicci 2006). The first *in vitro* study reporting these changes was conducted using SFN on prostate and colon cancer cell lines, where the inhibition of HDAC activity was accompanied by global increases in histone H3 and H4 acetylation on the promoter regions of p21 and bax genes, facilitating cell cycle arrest and apoptosis in the context of cancer chemoprevention (Myzak et al. 2004; Myzak et al. 2006). Later, the same group confirmed HDAC inhibition by SFN *in vivo*, using APC^{min} mice that had ingested 443 mg SFN/kg (~6 µmol SFN/day) for 70 days, observing the re-expression of p21 and bax genes that triggered cell cycle arrest and apoptosis in transformed cells and microadenomas, thereby suppressing polyp formation compared with controls (Myzak et al. 2006). Although the growing interest in the epigenetic regulation mediated by isothiocyanates has focused mainly in its HDAC inhibition activity, their potential chemopreventive mechanisms involving DNA methylation mechanisms remain relatively unknown.

On the other hand, selenium apart from being an important player in Se-anticarcinogenesis by way of its intermediary Se-metabolites or as an essential

component of antioxidant enzymes that act actively in the removal of reactive oxygen and nitrogen species, has been found to affect DNA methylation by influencing the activity of DNA methyltransferase enzymes (Fiala et al. 1998). However, the efficacy of different forms of selenium, particularly se-methylselenocysteine (SeMSC), in relation to cancer prevention and therapy through DNA methylation mechanisms remains unexplored. This form of selenium, which has been found to account for the anticarcinogenic effect of selenium-enriched broccoli, was also used in our research to decipher its potential to bring about epigenetic changes for cancer chemoprevention.

In the present study we quantitatively explored the effects of the isothiocyanates sulforaphane or iberin, either individually or in combination with selenium compounds such as selenite and SeMSC, to define the impact of these active food compounds on the epigenome, particularly the methylation status of 5 CpG islands ($p16^{INK4A}$, *ESR1*, *HPP1*, *APC* and *MGMT*) selected for their potential involvement in colon cancer.

3.0.1 Analytical Methods Used to Quantify DNA Methylation

There are multiple methods to study DNA methylation. Currently, the gold standard technique for fine mapping of methylated cytosines relies on a chemical reaction using sodium bisulphite ($NaHSO_3$), which can selectively deaminate cytosine but not 5-methylcytosine to uracil and subsequently, via polymerase chain reaction, to thymidines (Raizis et al. 1995), which leads to a primary sequence change in the DNA that will allow discrimination of cytosine from 5-methylcytosine (Figure 3.1A). Following conversion the sequence differences between a methylated and unmethylated cytosine can be interrogated by various methods.

Methylation-specific PCR (MSP) as a novel approach for rapid analysis of the methylation status of a CpG Island was initially identified in the mid-90s (Herman et al. 1996). In this method, PCR primers are designed to be complementary to completely methylated or completely unmethylated target DNA where methylated and unmethylated primers sets differ only in the CG position of the bisulphite converted primary sequence. Therefore, the methylation status could be discriminated by PCR with sequence-specific primers.

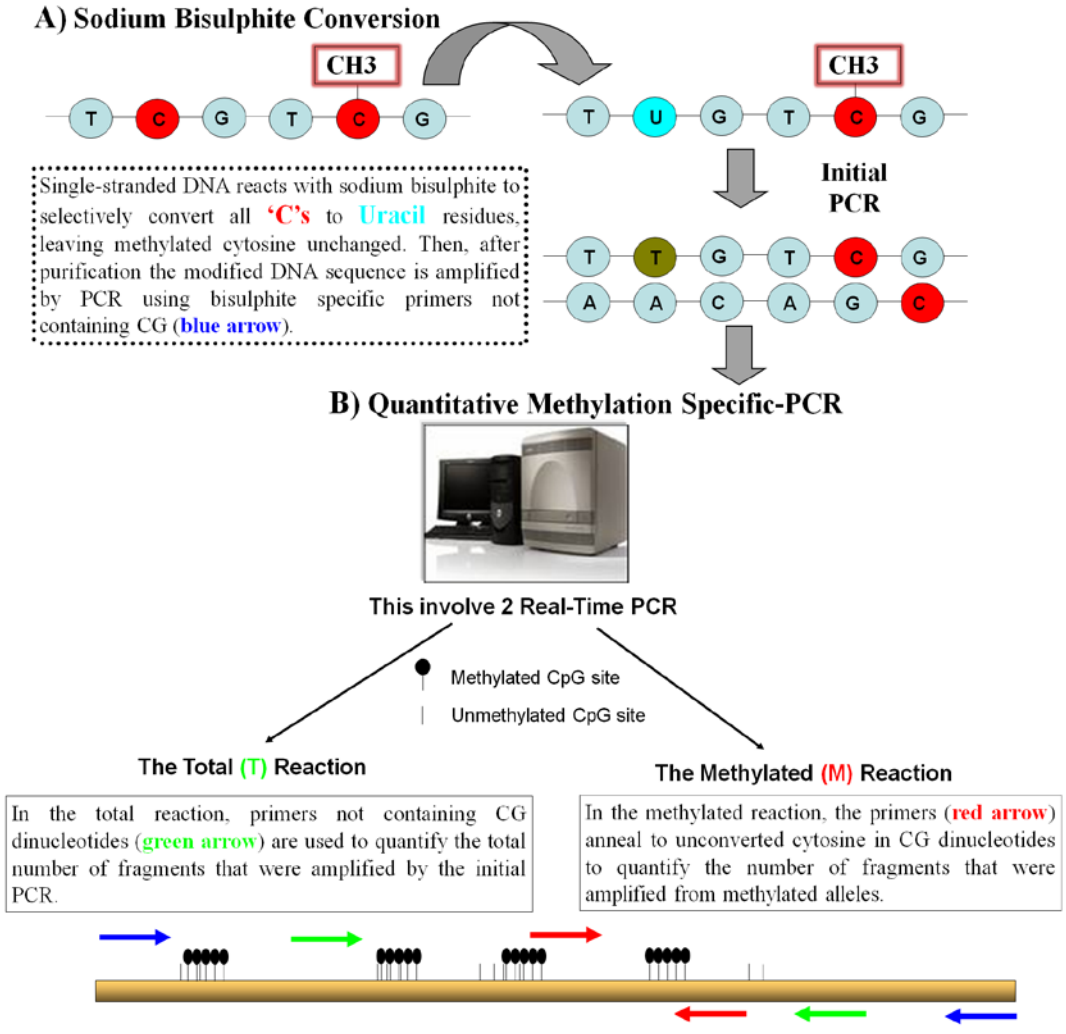


Figure 3.1 Schematic flowchart showing DNA methylation analysis by MethyLight technology. A) Firstly, samples are bisulphite converted. Here CpG Methylated cytosines are protected and remained unchanged, while unmethylated cytosines are deaminated to uracil after treatment with sodium bisulphite. B) Then, Quantitative Methylation Specific PCR or QMSP is applied to the initial PCR sample as shown above.

An improvement over the original MSP is MethyLight (Eads et al. 2000). This method determines the methylation status of a selected CpG island using fluorescence-based real-time PCR technology and requires no further manipulations after the PCR step and has been proposed for use in high-throughput methylation analysis, providing another expanding area in the DNA-methylation field because of its ability to detect minimal amounts of aberrant DNA methylation (Laird 2003). A modified version of this methodology, which utilises an initial PCR amplification of the CpG island of interest from bisulphite-modified genomic DNA prior to real-time PCR analyses, as outlined in Figure 3, was used in our study to interrogate the DNA methylation status of specific CpG promoters regions in Caco-2 cells and HCT116 (Figure 3.1B). However, as these assays rely on PCR of CG-rich DNA, the use of positive and negative control samples for methylated DNA (such as *in vitro* methylated DNA) and unmethylated DNA (noncancerous tissue) is always a requirement when using this approach. Another wide range of techniques for studying epigenetic changes in cancer cells, such as pyrosequencing, restriction landmark genomic scanning and others are explained in detail elsewhere (Esteller 2007).

New data highlighting the role of isothiocyanates and selenium in regulating the genome machinery through epigenetic mechanisms will be presented and discussed in this chapter on *in vitro* models of colon cancer.

3.1 MATERIALS AND METHODS

3.1.1 Materials

All materials and supplements used for cell culture and food compound treatments employed in this experimental research correspond to those used in work described Chapter 2. The colorectal adenocarcinoma cell line HCT116 was obtained from the American Type Culture Collection (Middlesex, UK). For bisulphite conversion, the QIAquick gel extraction kit and glycogen were purchased from Qiagen and Roche, UK respectively. ROX reference dye and SYBR green were obtained from Invitrogen, UK. Sodium bisulphite, hydroquinone, 5-Aza-2'-deoxycytidine, PCR primers and all other chemicals were purchased from Sigma-Aldrich (UK), unless otherwise stated.

3.1.2 Methods

3.1.2.1 Cell Culture and Treatments

Human epithelial colorectal adenocarcinoma Caco-2 cells obtained from the American Type Culture Collection (Middlesex, UK) at passage number 21 were seeded in 6-well plates (BD Falcon^{TM}) at a concentration of 8.0×10^4 cells/well in 3 ml of Dulbecco's Modified Eagle's Medium containing 4.5 g/L D-glucose and non-essential amino acid, supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 1% (v/v) of L-Glutamine and 1% (v/v) antibiotic solution consisting of penicillin (5000 units/ml), streptomycin (5000 µg/ml).Cells were maintained in a controlled atmosphere with 5% CO_2 at 37ºC in a HERAcell® 150 CO_2 incubator.

Methylation changes can be observed in culture, although in most cases, this process is slow and may occur over many generations (Razin and Cedar 1991). To allow these changes to take place Caco-2 cells were treated 24 h after seeding to allow cells to adhere to the bottom of the flask and then were supplemented for 4, 8 or 12 days with DMEM containing different concentrations of sulforaphane and iberin (ranging from 6 to 8 µM) or SeMSC and selenite (ranging from 0.2 to 5 µM), using three biological replicates per concentration in a final volume of 3 ml per well and replacing media with fresh dietary compounds every two days (Figure 3.2). Isothiocyanate compounds were dissolved in DMSO at appropriate concentrations so that, after addition to cells, all treatments and controls received 0.05% DMSO. Selenium compounds were dissolved in Milli-Q water and filtered through a 0.22 µm syringe filter. All stock and working solutions were aliquoted and stored at -80°C.

Caco-2 cells were passaged after reaching 70-80% confluence (usually on the 4^{th} day after treating the cells with food compounds), following the standard operating procedures for subculture of adherent cell lines. Briefly, medium was removed from the 6-well plates and the monolayer rinsed twice with 2 ml of sterilized Dulbecco's phosphate buffered saline (PBS). Then, 100 µl of Trypsin/EDTA (0.25% Trypsin, 1 mM EDTA) were added and mixed to ensure that the entire monolayer was covered with the Trypsin solution, the plate was incubated at 37^{o}C for 5-6 minutes until the cells were detached. After this, cells were re-suspended in 1.5 ml of fresh serum-containing medium to inactivate the Trypsin, pipetting the cells up and down until the cells were dispersed into a single cell suspension before proceeding to count the cells in the hemocytometer. All different treatments, including controls, were normalised by plating an appropriate cell number (8.0×10^4 cells/well) back into a new 6-well plate to adjust for any difference in growth, allowing 24 h for cell attachment before treatments were continued as mentioned above (Figure 3.2). The remaining cells were all processed the same day to extract DNA and RNA and samples were stored at -80°C until analysis.

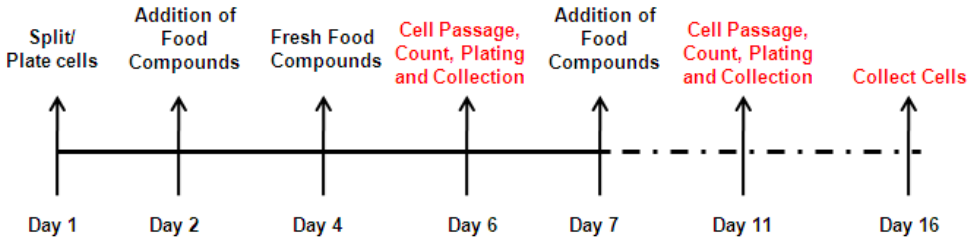


Figure 3.2 Schematic design of the schedule followed to treat cells to study DNA methylation changes in colon cancer cells. After day 6 and 11 the same steps carried out after day 1 were followed. The absolute numbers of days the cells remained in contact with media supplemented with food compounds for the days 6, 11 and 16 correspond to 4, 8 and 12 respectively.

In order to compare the DNA methylation results obtained with Caco-2 cells, another human-derived colon adenocarcinoma cell line (HCT116) was selected and cultured following the same standard operating procedures mentioned above for Caco-2 cells. In order to determine the number of cells to be plated to match the number of days of treatment for Caco-2 cells before confluence was reached, initially their growth was followed over time to obtain the normal cell growth characteristics of HCT116, which showed that seeding 6×10^4 cells/well in a 6-well plate would allow for the same cell growth interval.

3.1.2.2 Genomic DNA and RNA Isolation

In order to obtain the most reliable information from different cellular structural organizational levels using the same biological source, a simultaneous extraction of DNA and RNA from a single sample was carried out to allow for a more direct correlation between them. In this regard, DNA and RNA isolation from harvested Caco-2 and HCT116 cells was performed using the AllPrep DNA/RNA kit (Qiagen, UK), following the manufacturer's instructions. The NanoDrop spectrophotometer (Labtech International, UK) was used to quantify RNA concentration and purity by absorbance measurement at 260 and 280 nm.

3.1.2.3 Bisulphite Treatment of Genomic DNA

Bisulphite treatment of genomic DNA was originally carried out following a standard procedure (section 3.1.2.3.1). However, a high throughput method was later adopted (section 3.1.2.3.2). In order to compare the efficiency of the new adopted method with that previously used, samples were bisulphite converted using both methods in parallel and the global level of methylation of both samples was compared using a real-time PCR based assay for LINE-1 methylation, which showed no statistically significant effect of the bisulphite conversion method used (data no shown).

3.1.2.3.1 Standard Bisulphite Treatment

A bisulphite/hydroquinone solution containing 5 M sodium bisulphite and 140 mM hydroquinone was prepared fresh by adding 1.9 g of sodium bisulphite (Sigma, UK) to 3 ml of water. The sodium bisulphite was completely dissolved after 140 µl of 10 M NaOH and 0.7 ml of 0.75 M hydroquinone (Sigma, UK) was added, using regular vortexing until all the material was in solution. The genomic DNA was first denatured by alkaline treatment by mixing 17 µl of DNA (~400-500 µg) with 3 µl of 2 M NaOH (final concentration 0.3 M) and incubating at 37ºC for 15 min. After this, samples were exposed to the bisulphite/hydroquinone solution by adding 0.4ml to each sample, followed by an incubation at 50ºC for 4.5 hours in a Thermo Hybaid Omn-E PCR Thermocycler.

The bisulphite modified samples were purified by using a QIAquick gel extraction kit. Briefly, 1.26 ml of QG buffer was mixed with the sulphonated DNA and filtered through a QIAquick column using a vacuum manifold, followed by an additional 0.5 ml of QG buffer. The column was washed twice with 0.7 ml of PE buffer, placed in a collection tube and centrifuged for 5 min at full speed in a microcentrifuge to dry the membrane. DNA was eluted by pipetting 100 µl of Elution Buffer (EB) directly onto the filter, incubating at

RT for 1 min, and centrifuging for 1 min. Following this, an alkali treatment was applied to remove the sulphonate adducts by adding 3 µl of 10 M NaOH, and incubating at 37ºC for 15 min. The solution was neutralized by addition of ammonium acetate (NH_4OAc), pH 7, to a final concentration of 5 M and the DNA was ethanol precipitated by adding 525 µl of ethanol (1ul of glycogen was added as a carrier), and centrifuged at 4ºC for 30 min to pellet the DNA. Supernatant was discarded carefully with a pipette and the pellet was washed with 200 µl 70% ethanol, centrifuged for 5 min, dried and resuspended in 100 µl QIAquick elution buffer and stored at -20ºC.

3.1.2.3.2 Bisulphite Treatment: High-throughput

High-throughput bisulphite treatment of genomic DNA was performed with a the EZ-96 DNA Methylation^{TM} Kit (Zymo Research, USA), which combines bisulphite conversion and DNA clean-up in a 96-well plate format, in accordance with the manufacturer's instructions.

3.1.2.4 Initial PCR Amplification of CpG Islands

After obtaining the bisulphite-modified DNA, 5 µl were used as template in PCR reactions to amplify the CpG regions of the genes to be studied (*APC*, *ESR1*, *HPP1*, *p16* and *MGMT*). PCR reactions (20 µl) containing 10 µl of HotStarTaq master mix (Qiagen), 4 pmol of each forward and reverse primer (Table 3.1), and supplemented with $MgCl_2$ to 0.5 mmol/L, were subjected to the following cycling conditions: 1 cycle of 95ºC for 15 min, 35 cycles of 95ºC for 30s, annealing temperature (Table 3.1) for 1 min, 72ºC for 1 min followed by a 5 min extension at 72ºC. A No Template Control reaction (NTC) was included in every PCR assay to rule out reagent or water contamination. Products from the PCR reactions were used as templates for quantitative methylation-specific PCR (QMSP).

Table 3.1 Primer Sequences and Annealing Temperatures for Initial PCR and QMSP*

Gene	PCR**	Forward (5' – 3')	Reverse (5' – 3')	Annealing T (°C)
APC	Initial	GTTAGGGTTAGGTAGGTTGT	CCATAATAACTCCAACACCTA	59.5
	T	GGGTGTTATTGGAGATAGAAT	CCATAATAACTCCAACACCTA	59
	M	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	65
ESR1	Initial	GGGATGGTTTTATTGTATTAGATTTAAGGG	CTATTAAATAAAAAAAAACCCCCCAAAC	58
	T	GTAGTTTAAGATTTTTTTGGAG	AACTTACTACTATCCAAATACACCTC	58
	M	GCGAGGTGTATTTGGATAGTAGTAAGTTCGTC	GTAAAAAAAACCGATCTAACCGTAAACCTACG	66
HPP1	Initial	AGAGTTTTTTTTTTATGGTAGTAGTT	ACTCCCACAACACCATAACTA	56
	T	AGAGTTTTTTTTTTATGGTAGTAGTT	AACATCCAAAAACTAAACTCAA	58
	M	GTTTTTCGCGTTTTCGGCGT	ATCATCCCGCGAACGACGA	67
p16	Initial	GGTTTTTTTTAGAGGATTTGAGGGATA	CTACAAACCCTCTACCCACCTAA	62
	T	GGTTTTTTTTAGAGGATTTGAGGGATA	CCAACCAACCCCTCCTCTTT	63
	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCCGAACCGCGACCGTAA	74
MGMT	Initial	GGTTTGGGGGTTTTTGATTAG	CCTTTTCCTATCACAAAAATAATCC	60
	T	GGTATTAGGAGGGGAGAGATT	CCTTTTCCTATCACAAAAATAATCC	58
	M	CGTAGTCGTTTCGAGTAGGATC	GTACCCGAATAATCCTAAAAACG	61
LINE-1	U	TGTGTGTGAGTTGAAGTAGGGT	ACCCAATTTTCCAAATACAACCATCA	60
	M	CGCGAGTCGAAGTAGGGC	ACCCGATTTTCCAATACGACCG	60

***** Information adapted from (Belshaw et al. 2008).
**Initial= Primers used for an initial amplicon amplification of bisulphite converted samples; T= Using non CpG containing primers; M= Uses primers containing CGs; U= The unmethylated reaction for LINE-1 uses primers to non CpG containing sites.

3.1.2.5 Quantitative Methylation-Specific PCR (QMSP)

The primer pairs for the QMSP assay specifically anneal to bisulphite-converted genomic DNA. To determine the percentage of methylation, 2 real-time PCR reactions are carried out, in the first round (**T**otal reaction), primers flanking the target region are used to amplify the region of interest independent of the CpG content-i.e., the total reaction (T) primers should not include CGs and therefore should amplify previously methylated or unmethylated target sequences equally, whereas in the second round (**M**ethylated reaction) the primers bind to unconverted cytosine and contain CG dinucleotides to quantify the number of fragments that were amplified from methylated alleles (Figure 3.1).

The T and M reactions were carried out on 5 µl of the amplified CGI fragment obtained as described in section 3.1.2.4 (diluted 1.0×10^4 times in duplicate)

using 4 pmol of the appropriate primers (Table 3.1), 10 µl Immomix (Bioline, London, UK), 0.125µl of 0.01×SYBR green (Invitrogen, Paisley, UK), 0.4 µl ROX reference dye (Invitrogen) and supplemented with $MgCl_2$ and bovine serum albumin (BSA) to 0.5 mmol/L and 1 mg ml^{-1} respectively, and water to 20 µl. Following a 10-min hot start at 95ºC, PCRs were performed for 40 cycles of denaturing at 95 ºC for 30s, annealing temperature (Table 3.1) for 30s and extension at 72ºC for 30s using an ABI 7300 machine (Applied Biosystems, Warrington, UK). As a quality control procedure the NTC reaction from the initial PCR amplification (section 3.1.2.4) was run together with an additional NTC from the reagents and water used for the RT-PCR reaction to rule out contamination.

3.1.2.6 Global Methylation

In this study we quantitatively evaluated the global methylation status of the long interspersed nuclear element-1 (LINE-1), a highly repeated and widely interspersed human retrotransposon, in DNA isolated from Caco-2 cells. In normal somatic cells the bulk of methylcytosines, which constitutes a substantial portion (~17%) of the human genome, is found in repetitive sequences such as LINE-1. Within these regions cytosines are heavily methylated to help maintain chromosomal integrity, by preventing chromosomal instability, translocation and gene disruption through the reactivation of endoparasitic sequences (Esteller 2007). In line with this organisation, previous studies have established the overall decrease in methylation found in genomic repetitive sequences as a marker of the 5-methylcytosine level in several malignancies including carcinoma of urinary bladder, liver, prostate, and colon (Chalitchagorn et al. 2004; Schulz 2006; Ogino et al. 2008).

The assay for LINE-1 methylation was described previously (Iacopetta et al. 2007) and involves 2 real-time PCRs; one (the unmethylated (U) reaction) uses primers to non CpG containing sites to quantify the number of unmethylated

LINE-1 elements, whilst the other (the methylated (M) reaction) uses primers containing CGs to quantify the number of methylated alleles (Table 3.1). The bisulphite-modified genomic DNA was diluted by adding 5 µl of bisulphite-modified DNA to 45 µl 20 mM TrisHCl pH8 and the real-time PCR conditions used correspond to those used above for the QMSP assay.

3.1.2.7 Standard Curve: Quantification of Methylated Alleles

A standard curve obtained from a cloned fragment of the bisulphite-modified gene of interest from a completely methylated allele at known concentrations was run in every assay of QMSP and global methylation to relate C_T values to DNA concentration. The percentage of methylation in a sample was calculated using the following equations:

QMSP assay:

$$\%Methylation = \frac{\mathrm{M}}{\mathrm{T}} \times 100$$

Where M is the number of methylated copies of the gene and T is the total number of PCR fragments (methylated and unmethylated) present in the pool.

Global Methylation assay:

$$\%Methylation = \frac{\mathrm{M}}{\mathrm{U} + \mathrm{M}} \times 100$$

Where M and U corresponds to the number of methylated and unmethylated copies of the genes present in the pool respectively.

3.1.2.8 Preparation of Controls for DNA Methylation

3.1.2.8.1 Non-cancerous Tissue

Placental DNA (Sigma-Aldrich) and a noncancerous colon tissue biopsy of a young person (with ethical approval for research use) were used for controls. The frozen colon tissue was sliced and transferred to a 1.5 mL microcentrifuge tube to be weighed (obtaining ~25 mg). The genomic DNA of the colon specimen was isolated using the GenElute™ Mammalian Genomic DNA Miniprep (Sigma, UK) following the manufacturer's instructions.

M.SssI methylase (New England Biolabs, UK) was used to artificially methylate 1.5 µg of placental DNA (Sigma-Aldrich) or 1.5 µg of human noncancerous colon tissue DNA. The reaction was carried out by combining 2 µl of M.SssI methylase, 20µl 10×M.SssI buffer, 2 µl of S-adenosylmethionine (SAM) (32 mM) and water to 200 µl. Initially samples were incubated containing only 1 µl of SAM; an additional 1 µl was added after the first hour to give a final SAM concentration of 320 µM and incubation was continued at 37ºC for a further 1 hour. A similar reaction was prepared with all the previous reagents except the M.SssI methylase to serve as an additional, mock-methylated control. Samples were ethanol precipitated and re-suspended in 17 µl of EB for bisulphite modification.

3.1.2.8.2 5-Aza-2'-Deoxycytidine Treatment

As an additional control for our experiments cells were treated with 500 nM of the pharmaceutical compound 5-Aza-2'-deoxycytidine. This compound is a ring analogue of the pyrimidine nucleoside cytidine and differs by having a nitrogen atom in place of a carbon group and is widely used as a DNA methylation inhibitor to induce gene expression and cellular differentiation (Figure 3.3). In order to exert its biological function the drug gets incorporated

into DNA. DNA substituted with this cytidine analogue forms covalent adducts with cellular DNA methyltransferase (DNMT), thus depleting the cell of enzyme activity and promoting demethylation of genomic DNA as a secondary consequence (Juttermann et al. 1994).

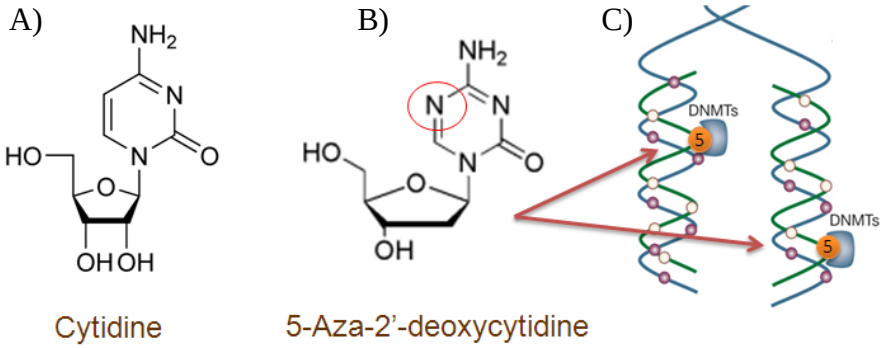


Figure 3.3 Chemical structure of A) Cytidine and B) 5-Aza-2'-deoxycytidine, highlighting in red their main structural difference. Figure C) shows graphically how 5-Aza-2'-deoxycytidine acts to inhibit *DNMT*s. This inhibitor once incorporated into the DNA form covalent bonds with the major DNA methyltransferase 1 depleting the enzyme and resulting in passive demethylation over successive rounds of DNA replication. Figure C) was adapted from (Egger et al. 2004)

3.1.2.9 Quantification of DNA Methyltransferases by Real-Time PCR

Total RNA extracted from the same biological sample used to extract DNA for DNA methylation analyses (section 3.1.2.2**)** was used to generate cDNA according to the steps mentioned in Chapter 2 (section 2.1.2.3.3). Quantitative real time PCR was performed to measure the mRNA levels of the DNA methyltransferases, DNMT1, DNMT3A and DNMT3B, using the ABI 7300 Real-Time PCR System (Applied Biosystems, Warrington, UK). Primers and probes (Table 3.2) were labelled with a 5´ reporter dye (FAM) and 3´ quencher dye (TAMRA). Real-time PCR reactions were carried out in a 96-well optical plate in a 20 µl final reaction mixture consisting of 5 µl of template, 4 pmol primers and 2 pmol of the appropriate probe, 10 µl Immomix (Bioline, London,

UK), 0.4 µl ROX reference dye (Invitrogen), supplemented with $MgCl_2$ and BSA to 0.5 mmol/L and 1 mg ml^{-1} respectively, and water to 20 µl. RT-PCR quantification was performed following a 10-min hot start at 95ºC, followed by 40 cycles at 95 ºC for 30s and 60 ºC for 60s. Reactions were carried out using two technical replicates for every biological replicate and data were normalized against an invariant endogenous control, *18S* ribosomal RNA. The threshold cycle number (Ct) obtained was converted into fold of relative induction using the ΔΔCt method following the equation: $E = (10^{-1/slope} - 1) \times 100$ (Livak and Schmittgen 2001)**.**

Table 3.2 Primers and Probes Sequences Used for Real-Time PCR

Gene		Sequence (5' – 3')
DNMT1	Forward	CAGCCAACAGAGGACAACAA
	Reverse	CCGGCTATCCAGGTCCTC
	Probe	Universal LibraryPprobe #1 (CCTGGAGC)
DNMT3A	Forward	CAATGACCTCTCCATCGTCAAC
	Reverse	CATGCAGGAGGCGGTAGAA
	Probe	AGCCGGCCAGTGCCCTCGTAG
DNMT3B	Forward	CCATGAAGGTTGGCGACAA
	Reverse	TGGCATCAATCATCACTGGATT
	Probe	CACTCCAGGAACCGTGAGATGTCCCT
18S rRNA	Forward	GGCTCATTAAATCAGTTATGGTTCCT
	Reverse	GTATTAGCTCTAGAATTACCACAGTTATCCA
	Probe	TGGTCGCTCGCTCCTCTCCCA

3.1.2.10 Statistics

Statistical analysis was carried out with SPSS 16 statistical program. Results are expressed as means ± SD. Statistical comparisons were made using one-way ANOVA with Dunnett's *post hoc* test.

3.2 RESULTS AND DISCUSSION

3.2.1 Effect of Iberin and SeMSC on Epigenetic Modifications: A DNA Methylation Screening in Caco-2 cells

In the present study CpG island methylation analysis was conducted in Caco-2 cells for key genes that have been shown to be involved in colorectal neoplasia and to be affected by aberrant CpG island methylation. Numerous studies have reported an effect of dietary constituents in different cell lines and animal models on the methylation status of genes involved in the cancer process. Among such studies, effects of isothiocyanates on aberrant gene-specific methylation have been reported. In prostate cancer cells, reactivation of the silenced glutathione S-transferase gene (*GSTP1*) was observed following treatment with phenethyl isothiocyanate (PEITC) (Wang et al. 2007). In oesophageal squamous cell lines, sulforaphane (SFN) treatment led to the reactivation of the *p16* and *MGMT* genes, which was further enhanced by a combination of both genistein and SFN (Fang et al. 2005).

Many studies have focused on SFN, but here the use of the analogue isothiocyanate iberin, which contains one alkyl group less than SFN, was investigated to gain insights into the mechanisms involved in the regulation of methylation. Synergistic effects of co-treatment with Se-methylselenocysteine were also explored.

Initially, we conducted a pilot DNA methylation screening of 5 genes: $p16^{INK4A}$, *ESR1* (Oestrogen receptor α), *APC* (Adenomatous polyposis coli), *MGMT* (O6-methylguanine-DNA methyltransferase) and *HPP1* (Hyperplastic polyposis protein 1). These genes were selected based on their involvement in cell growth control, differentiation, migration, apoptosis and DNA damage repair in colon cancer.

The data revealed that the CpG islands of *p16*^{INK4A} and *ESR1* in control samples (DMSO treated) were heavily methylated (~80%), whereas the percentage of methylation for *HPP1* and *APC* genes in the controls were 46%

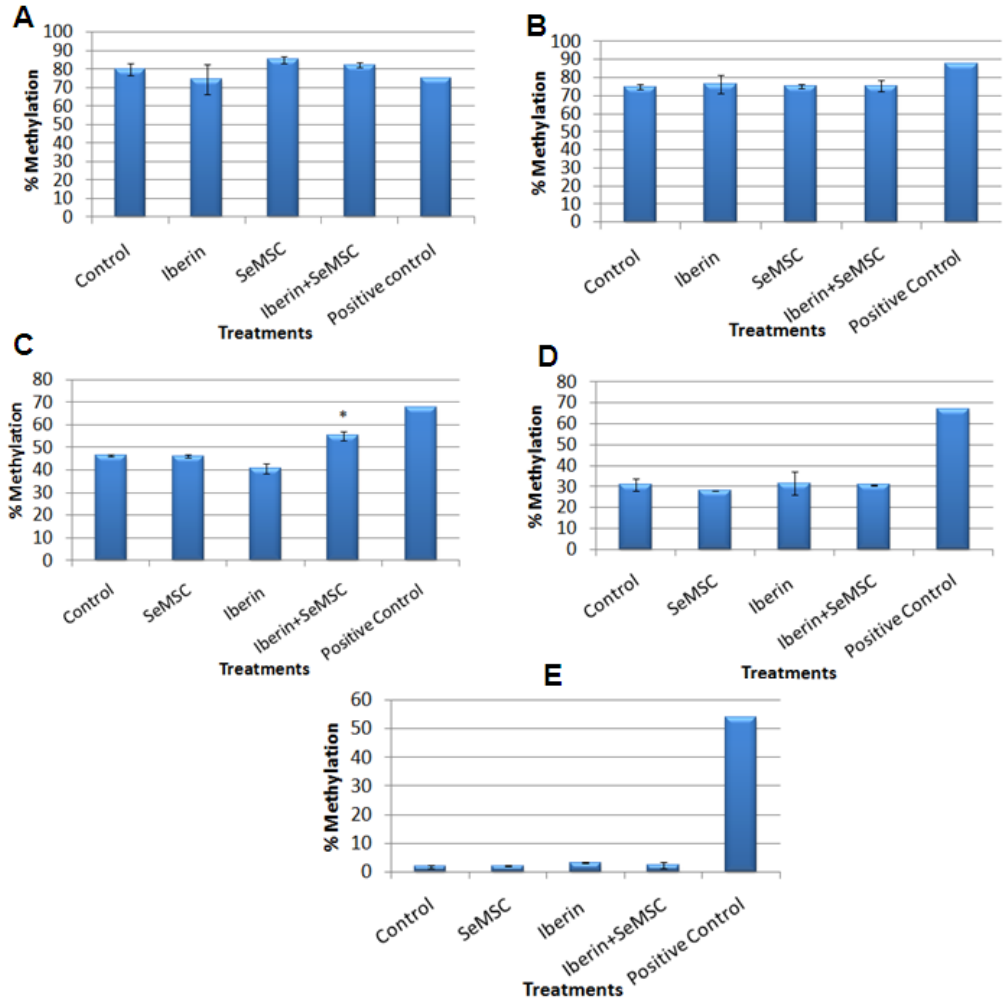


Figure 3.4 DNA methylation status of A) p16 ^{INK4A}; B) ESR1; C) HPP1; D) *APC*; and E) MGMT CpG islands. Caco-2 cells were exposed to DMSO (control), 6 µM of iberin and/or 200 nM of SeMSC for 4 days before genomic DNA was isolated and bisulphite converted to evaluate its effect at the DNA methylation level. The positive control represents DNA from human noncancerous colon that was artificially methylated using CpG methyltransferase (M.SssI) as stated in the method section. Significant differences from control are indicated (**P*<0.05).

and 31%, respectively. The *MGMT* CpG island was not methylated (Figure 3.4 A-E). However, it was observed that the methylation status of the vast majority of CpG islands were unaffected after incubating Caco-2 cells for 4 days with iberin and/or SeMSC when compared with control. Only the methylation status of the *HPP1* gene was increased significantly by 9% by the iberin+SeMSC

treatment. Although significant ($P<0.05$), this increase was not considered relevant at first, given the fact that the other genes did not show such an increase in DNA methylation under this treatment.

In a study carried out to profile DNA methylation patterns of 70 established cancer cell lines using 15 candidate genes (among them two of our target genes: *p16*^{INK4a} and *MGMT*), although only a qualitative approach was used to measure CpG island methylation (Methylation-Specific PCR), it was found within the colon cancer cells studied that 100% (11/11) were methylated for the *p16*^{INK4a} gene and 55% (6/11) were methylated for *MGMT* (Paz et al. 2003). In this study, *MGMT* was unmethylated in the Caco-2 cell line, in agreement with the results obtained here (Figure 3.4E). With regards to level of methylation found in the *APC* promoter region in control samples, it is a frequent epigenetic alteration in colorectal cancer and the level of methylation has been shown to change depending on the stage of the disease and according to the cell line studied (Deng et al. 2004; Chen et al. 2005)

3.2.2 DNA Methylation Dynamics Over Time: Influence of Iberin and/or SeMSC on DNA methylation Changes in a Time-Dependent Manner

To explore the possibility that increased incubation times with the food compounds may elicit an effect on DNA methylation, the experiment was repeated but with two extra time points added (day 11 and 16 depicted in Figure 3.2). In this experiment only *p16* ^{INK4A} and *ESR1* were chosen for further analysis, as these genes were found to have the highest level of methylation in control samples (Figure 3.4 A, B). However, as demonstrated in Figure 3.5 no additional changes were observed when Caco-2 cells were exposed to iberin and/or SeMSC for up to 12 days (Figure 3.5 A, B). Also, in an attempt to identify potential global (genome-wide) epigenetic changes, the methylation status of LINE-1 elements, a surrogate marker of genome-wide methylation,

was measured. The results showed that methylation of LINE-1 in the control group was low (65%), which is a common characteristic of cancer cells. The

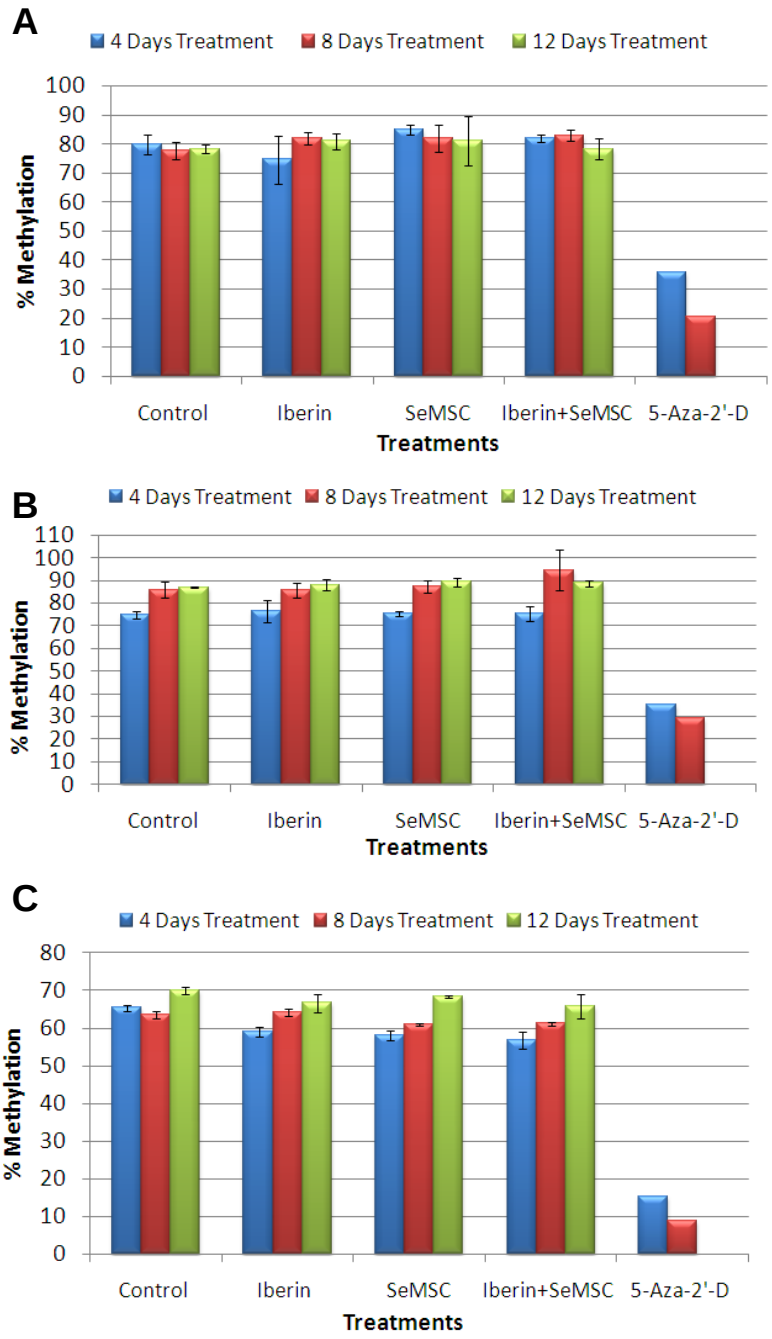


Figure 3.5 DNA methylation status of A) *p16* ^{INK4A}; B) *ESR1*; and C) LINE-1. Caco-2 cells were exposed to DMSO (control), 6 µM of iberin and/or 200 nM of SeMSC for 4, 8 and 12 days before genomic DNA was isolated and bisulphite converted to evaluate its effect at the DNA methylation level. As a positive control 500 nM of the *DNMT* inhibitor 5-Aza-2'-deoxycytidine was used. However, because of 5-Aza-2'-deoxycytidine known cell toxicity when used for several days in cell culture this treatment was only continued until day 8.

treatment of cells with the *DNMT* inhibitor 5-aza-2'-deoxycytidine resulted in an expected 80, 70 and 90% reduction in *p16 INK4A ESR1* and LINE-1 methylation respectively after 8 days of treatment. However, no significant changes in DNA methylation were seen for LINE-1 when cells were exposed to the dietary agents (Figure 3.5C).

3.2.3 Effect of Time and Dose of Different Isothiocyanates and Selenium Forms on DNA Methylation and DNA Methyltransferase Expression

In order to investigate whether the form and/or concentration of isothiocyanates and selenium used were not effective in inducing DNA methylation changes, sulforaphane and selenite were also included within the experimental design. A potential effect on DNA methyltransferase gene expression (*DNMT1*, *3A* and *DNMT3B*) was also investigated. Cells were treated for up to 12 days with SeMSC or selenite (0.2 to 5 µM), SFN (8 µM) alone or in combination with SeMSC (1 µM). Initially, only the first two time points (4 and 8 days) were evaluated to screen the DNA methylation status of the genes of interest. However, as shown in Figure 3.6, no effects were observed for the methylation of *p16*^{INK4A} or *ESR1*, and only cells treated with 5-aza-2'-deoxycytidine responded significantly. Similar to our results, Davis et al. (2000) found that the tumour suppressor gene *p16*^{INK4A} was completely methylated regardless of treatment with 1 or 2 µM of selenite in Caco-2 cells. But, in contrast, the methylation of the *p53* promoter region decreased when cells were cultured in the absence of selenite.

The methylation status of LINE-1 (Figure 3.7) was equally unresponsive to the different concentrations or incubation times compared with control samples, which showed significant hypomethylation (~53-58%) in this cell line. Figure 3.6 and 3.7 also show the evaluation of a mock-methylated control sample from normal human colon tissue, in which *p16*^{INK4A} and *ESR1* are not

methylated and LINE-1 is hypermethylated as would be expected in "normal" tissues. Moreover, we have included as an additional control for our experiments human placental DNA, which is known to be globally hypomethylated (Reiss et al. 2007). This was confirmed in the mock-methylated placental DNA sample in Figure 3.7, where a 30% methylation value was obtained, corroborating the accuracy of this assay.

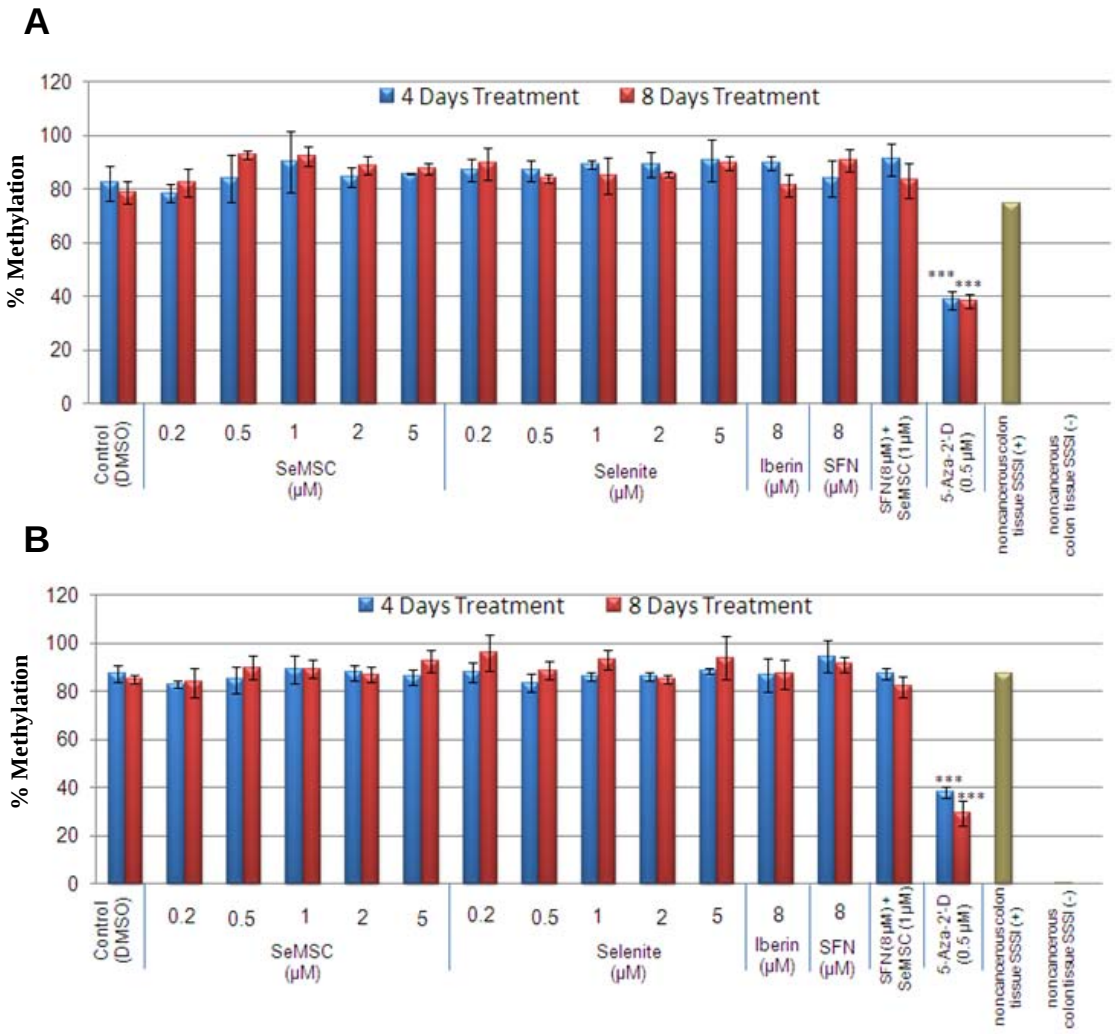


Figure 3.6 DNA methylation status of A) *p16* ^{INK4A} and B) *ESR1* in Caco-2 cells treated with SeMSC, selenite, iberin, SFN, SFN+SeMSC and 5-Aza-2'-D. Cells were treated with control (DMSO) and with concentrations of isothiocyanates and/or selenium shown above for 4 and 8 days, before genomic DNA was isolated and bisulphite converted. As a positive control, 500 nM of the *DNMT* inhibitor 5-Aza-2'-deoxycytidine was used. Additional controls included DNA from human noncancerous colon that was artificially-methylated (+) or mock-methylated (-) using CpG methyltransferase (M.SssI) as stated in the method section. Significant differences from control are indicated (***P<0.001).

SeMSC-treatment for 4 or 8 days cells did not change the expression levels of the 3 DNMTs studied (Figure 3.8A-C). However, some significant effects were observed after selenite treatment for 4 days, particularly for DNMT3A and DNMT3B. But, this observed change did not follow a dose- or time-dependent pattern. Similarly, of the different isothiocyanates studied, iberin exhibited statistically significant but transient up-regulation of the DNMT genes after 4 days of treatment, which did not continue to 8 days. However, this increased expression did not correlate with an increased level of methylation of the *p16*^{INK4A} or *ESR1* promoter regions nor of LINE-1. This is compared with the significant effect on the methylation status of these genes (Figure 3.6 A, B) and LINE-1 (Figure 3.7) following treatment with 5-aza-2'-deoxycytidine, which decreased the expression of the DNMTs after 8 days of treatment (Figure 3.8A-C). The treatment with SFN+SeMSC did not have a synergistic effect at the DNA methylation level, confirming that neither iberin nor sulforaphane in combination with an organic form of selenium (Figure 3.5 and 3.6 respectively) modify DNA methylation status of the genes studied in Caco-2 cells.

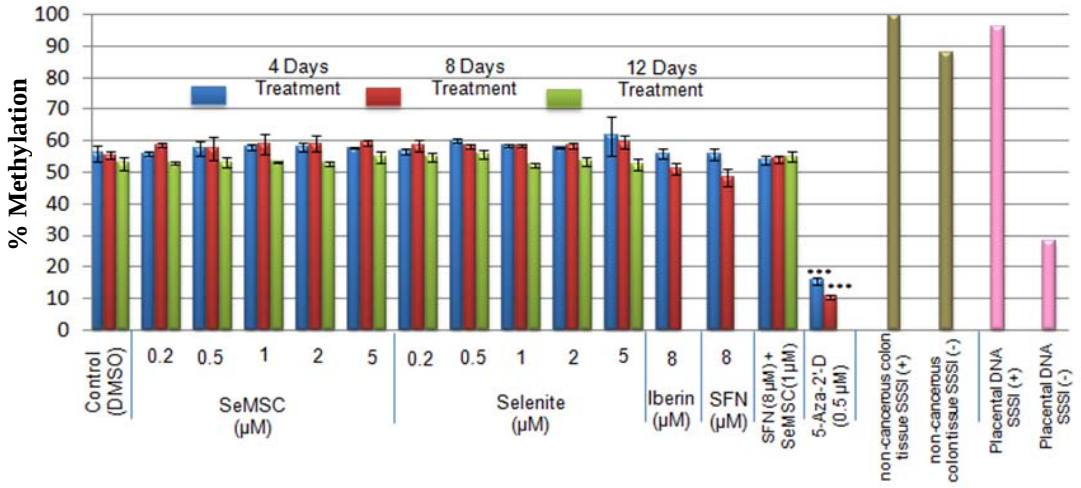


Figure 3.7 DNA methylation status of LINE-1 in Caco-2 cells treated with SeMSC, selenite, iberin, SFN, SFN+SeMSC and 5-Aza-2'-D. Cells were treated with control (DMSO) and with concentration of isothiocyanates and/or selenium shown above for 4, 8 and 12 days, before genomic DNA was isolated and bisulphite converted. As a positive control, 500 nM of the *DNMT* inhibitor 5-Aza-2'-deoxycytidine was used. Additional controls included DNA from human noncancerous colon and human placenta that was artificially-methylated (+) or mock-methylated (-) using CpG methyltransferase (M.SssI) as stated in the method section. The treatments iberin, sulforaphane and 5-Aza-2'-deoxycytidine were only continued until day 8 due to a decrease in cell viability. Significant differences from control are indicated (***P<0.001).

To exclude the possibility that an effect might have taken place on the remaining time point (Day 12), it was decided to examine first the methylation status of LINE-1 (Figure 3.7) and the expression of the different *DNMT*s

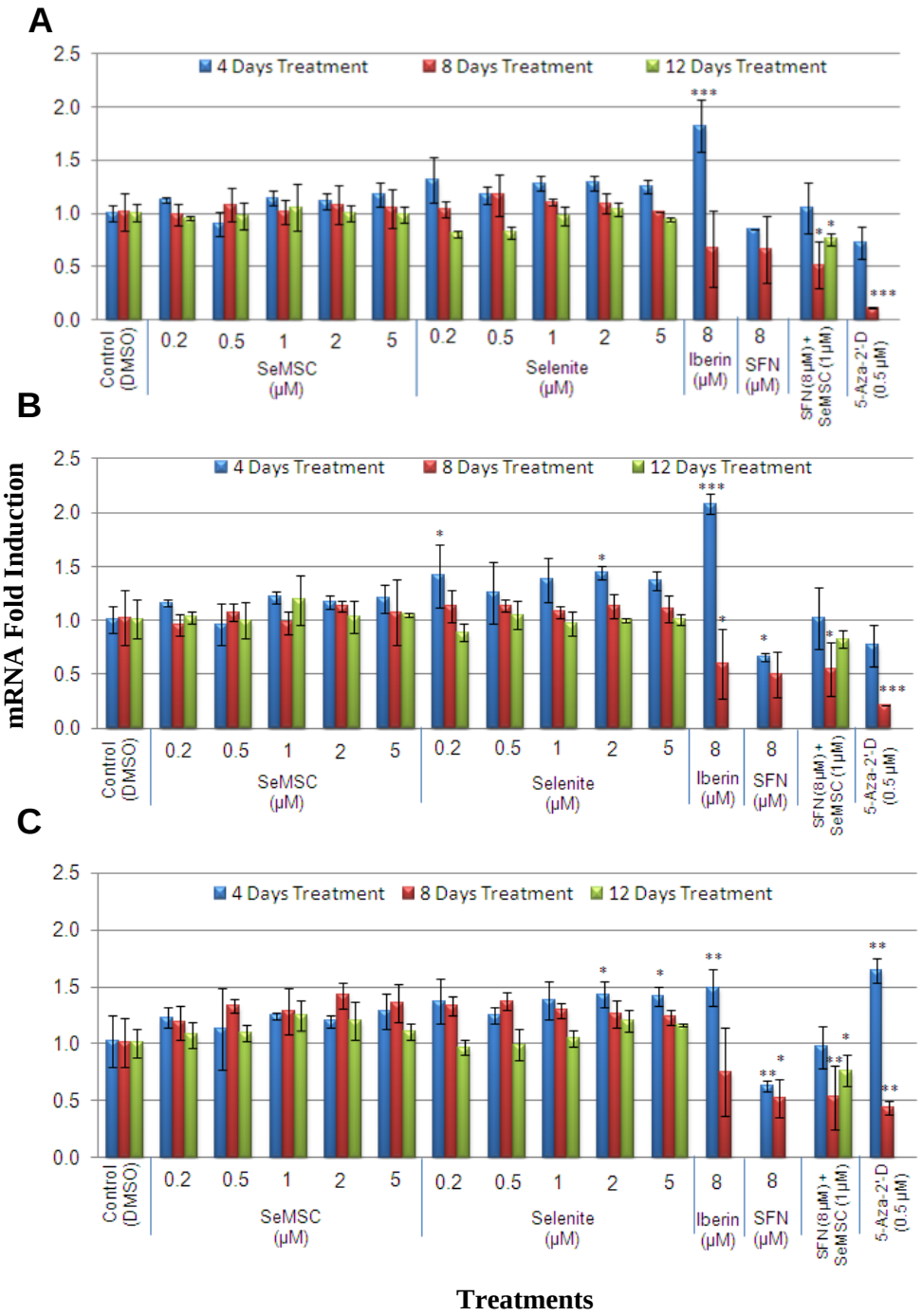


Figure 3.8 Effect of SeMSC, selenite, iberin, SFN, SFN+SeMSC and 5-Aza-2'-D on DNMTs mRNA: A) DNMT1; B) DNMT3A; C) DNMT3B in Caco-2 cells. Cells were treated with control (DMSO) and with concentration of isothiocyanates and/or selenium shown above for 4, 8 and 12 days. The treatments iberin, sulforaphane and 5-Aza-2'-deoxycytidine were only continued until day 8 due to a decrease in cell viability. Significant differences from control are indicated (**P*<0.05; ***P*<0.01; ****P*<0.001).

(Figure 3.8), but no significant changes were observed. It was therefore considered unnecessary to proceed with the CpG island methylation analyses of *p16*^{INK4A} and *ESR1* for this time point.

3.2.4 DNA Methylation Status of HCT116 Cells

HCT116 cell line was used to address whether the lack of effect of selenium and/or isothiocyanates on DNA methylation in Caco-2 cells is a common phenomenon that occurs in other colorectal cancer cell lines. Previous studies have shown that exposure of human colon carcinoma HCT116 cells to phenylenebis-(methylene)-selenocyanate (*p*-XSC) (another organoselenium compound) for 24 h caused DNMT inhibition with an IC_{50} of ~20 µM. In addition, the same study found that the *in vitro* effects of the chemopreventive compounds, sodium selenite, benzyl selenocyanate (BSC) and *p*-XSC on the activity of DNMT in nuclear extracts of a human colon tumour resulted in a concentration dependent inhibition of activity with an IC_{50} of 3.8, 8.1 and 5.2 µM respectively (Fiala et al. 1998). However, the previously described research only measured DNMT enzyme activity and did not investigate the impact of this inhibited enzyme activity on DNA methylation levels. Therefore, in an attempt to expand the results obtained by Fiala et al., and in order to corroborate our results, HCT116 cells were exposed to SeMSC or selenite (2 µM) and sulforaphane or iberin (6 µM) for 8 days. Synergistic effects between the compounds were also explored. Although the treatment with SeMSC and/or isothiocyanates was successfully completed for 8 days, the cells supplemented with selenite were only continued until day 4 as a reduction in cell viability was observed. As a consequence, an additional treatment with 500 nM of selenite was set up for 4 days in order to identify potential epigenetic changes that may have been masked as a result of the sodium selenite toxicity. It is important to mention that such toxicity with selenite was not found in Caco-2 cells in which doses of up to 5 µM of selenite were tolerated.

In the present investigation we have found that the level of methylation of our target genes in HCT116 control cells corresponds to 40-50% and 80-90% for *p16*^{INK4A} and *ESR1*, respectively (Figure 3.9A, B). Whereas most colon cancer cell lines have been shown to contain only hypermethylated *p16*^{INK4A} alleles (as observed in Figure 3.6A for Caco-2 cells), the cell line HCT116 has been reported to be heterozygous for a frameshift mutation in the first exon of *p16*^{INK4A} resulting in a premature stop codon (Okamoto et al. 1994). Consequently, the methylation status of the CpG island in the promoter region of this gene has been found to be ~50%, which corresponds only to the wild-type allele of the *p16*^{INK4A} as the remaining mutated allele is unmethylated (Myohanen et al. 1998). Importantly, the percentage of methylation identified in the control sample (Figure 3.9A) matched accurately the methylation status of *p16*^{INK4A} previously reported. Similarly, other reports have identified the *ESR1* CpG island to be hypermethylated in the HCT116 cell line (Xiong and Laird 1997; Eads et al. 2000) as found here (Figure 3.9B). In relation to the global methylation status of HCT116 (Figure 3.9C), two studies have identified that the methylation status of LINE-1 is between 66 and 77% (Yang et al. 2004; Aparicio et al. 2009). Although the LINE-1 methylation levels found in our study are higher (90%) than those reported earlier for HCT116, this percentage was consistently higher throughout the different treatments and more importantly the results obtained from the different controls used (normal colon tissue, placental DNA and 5-aza-2'-deoxycytidine) validated our findings, ruling out any isolated problem within our technology (Figure 3.9C) and suggest that the difference between LINE-1 methylation measured here and in previous reports may reflect the differences in the methodologies used for its quantification.

.

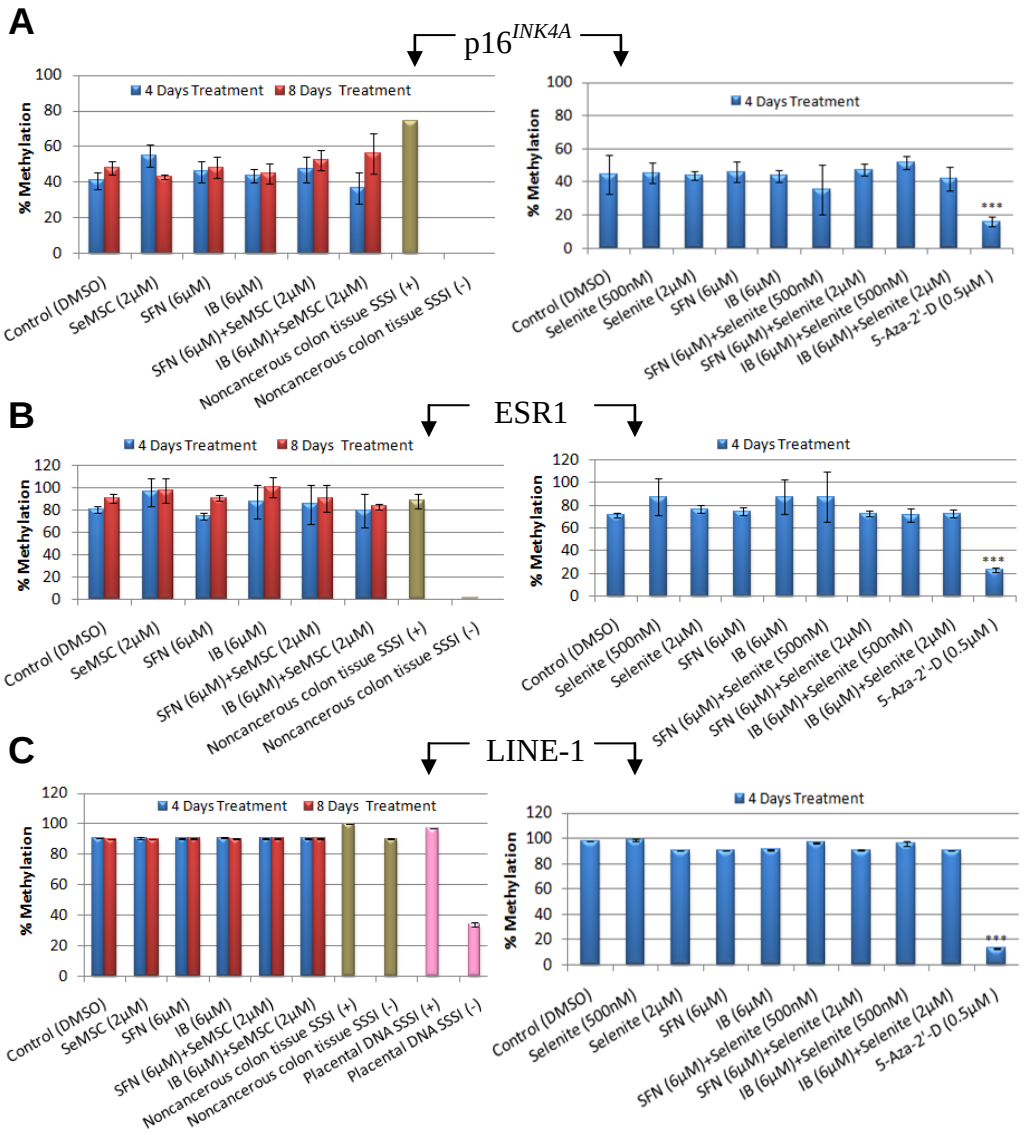


Figure 3.9 DNA methylation status of A) *p16* ^{INK4A}, B) *ESR*1 and C) LINE-1 in HCT116 cells treated with SeMSC, selenite, iberin, SFN, and its combination. Cells were treated with control (DMSO) and with concentration of isothiocyanates and/or selenium shown above for 4 and 8 days, before genomic DNA was isolated and bisulphite converted. The graph on the left side represents HCT116 cells supplemented with SeMSC and/or ITC, whereas the one on the right side includes the selenite and/or ITC treatments. As a positive control, 500 nM of the *DNMT* inhibitor 5-Aza-2'-deoxycytidine was used. Additional controls included DNA from human noncancerous colon and placental DNA (the latter used only for LINE-1) that was artificially-methylated (+) or mock-methylated (-) using CpG methyltransferase (M.SssI) as stated in the method section. Significant differences from control are indicated (***P<0.001).

The results from the current study with HCT116 cells suggest that the different forms of selenium and/or isothiocyanates used did not affect the gene-specific nor global methylation status when compared with untreated cells. A

significant increase in the level of DNMT1 and DNMT3B mRNA expression was observed for some of the treatments of HCT116, particularly at day 4 (Figure 3.10A, C), which was also demonstrated in the iberin-treated Caco-2 cells (Figure 3.8). However, this increase in expression was also transient and did not parallel an increase at the gene-specific or global LINE-1 methylation level. Interestingly, previous studies have found that most cases of frequent hypermethylation of CpG islands in human colorectal tumours do not result from a simple transcriptional up-regulation of any of the three known DNMT genes indicating, perhaps, that one or more of these genes are up-regulated post-transcriptionally (Eads et al. 1999). Moreover, previous studies have demonstrated that the levels of DNMT1 mRNA are regulated with the cell cycle at the post-transcriptional level, with a peak in S phase attributable to the change in the mRNA stability (Szyf et al. 1991; Szyf and Detich 2001). Importantly, isothiocyanates and selenium have been shown to posses anticarcinogenic properties by modulating the cell cycle (Visanji et al. 2004; Zeng and Combs Jr 2008) and it might be the case that the altered level of mRNA expression seen responds to changes in cell cycle progression caused by the food constituents. In addition, others have shown that the PI3K/PKB pathway elevates cellular DNMT1 protein expression independently of mRNA levels (Sun et al. 2007).

The regulation of DNMT3A and 3B has not been studied as extensively as that of DNMT1, but it has been shown that DNMT3B mRNA is also regulated with the cell cycle, and its expression profile is similar to that of DNMT1 (Robertson et al. 2000). This observation correlates well with the data presented here with similar patterns of mRNA expression between DNMT1 and DNMT3B observed with the different treatments (Figure 3.10 A, B).

Previous studies with DNMTs have also tried to establish associations between their level of expression at the transcriptional and translational level with pathologies. For instance, Saito and co-workers (2001) found no significant relationship between DNMT mRNA levels and DNA hypermethylation of CpG

islands in hepatocellular carcinomas, but the same group later reported a significant relationship between DNMT1 protein expression and the malignant

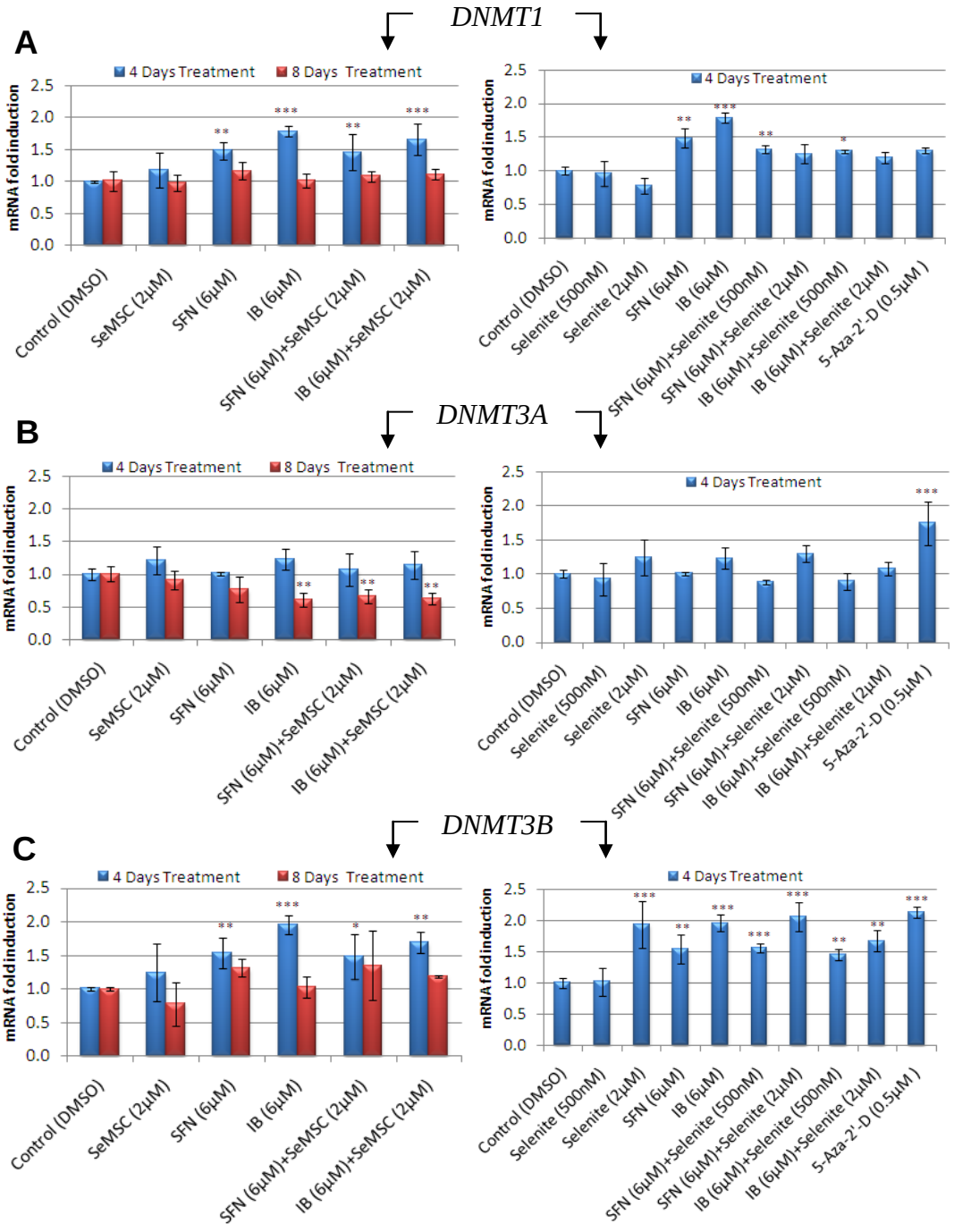


Figure 3.10 Effect of SeMSC, selenite, iberin, SFN and its combination on DNMTs mRNA: A) DNMT1; B) DNMT3A; C) DNMT3B in HCT116 cells. Cells were treated with control (DMSO) and with concentration of isothiocyanates and/or selenium shown above for 4 and 8 days. The graph on the left side represent HCT116 cell supplemented with SeMSC and/or ITC, whereas the one on the right side includes the selenite and/or ITC treatments. As a positive control, 500 nM of the *DNMT* inhibitor 5-Aza-2'-deoxycytidine was used. Significant differences from control are indicated (**P*<0.05; ***P*<0.01; ****P*<0.001)

potential and poor prognosis of human hepatocellular carcinomas (Saito et al. 2003), reflecting the importance of measuring both mRNA and protein level of DNMTs to accurately determine its expression status in cancer cells.

Overall, the present study clearly demonstrates that the food compounds used in this study did not reverse the DNA methylation status of 4 important genes involved in carcinogenesis (with the exception of *MGMT* that was shown to be unmethylated). Moreover, this lack of effect on aberrant DNA methylation patterns, including CpG island hypermethylation and repetitive region hypomethylation, was also confirmed when dose- and time-dependent experiments were conducted. Other studies in LNCaP prostate cancer cells have demonstrated that 1.5 μM of selenite treatment for 4 days did not cause significant changes in DNMT mRNA, but after 8 days a significant reduction of DNMT1, DNMT2 and DNMT3A mRNAs were seen with a concomitant reduction of DNMT1 at the protein level and reactivation of the silenced *GSTP1* gene (Xiang et al. 2008). However, as shown here, colon cancer cells did not respond in terms of altered DNA methylation patterns after 12 days of continuous treatment using up to 5 μM of an organic or inorganic form of selenium. Although there were some effects on the expression of the DNMTs studied, including an increase in the expression of these enzymes in both cells lines and particularly with the selenite form. However, this up-regulation of expression of DNMT1 (Figure 3.10A) and DNMT3B (Figure 3.10C), after 4 days of treatment, was transient and returned to the level of the control group after 8 days of treatment. Additionally, after 8 days of treatment with iberin or the ITCs+SeMSC group a significant down-regulation of DNMT3A mRNA was observed (Figure 3.10B). In contrast to Caco-2 cells, HCT116 cells did not show a down-regulation of the different DNMTs mRNA levels after 4 days of treatment with the DNMT inhibitor 5-aza-2'-deoxycytidine, reflecting differences in DNA methylation regulation among cell types.

Reports from other investigators have indicated that dietary selenium can inhibit DNMT1 activity *in vitro* from rat liver, Friend erythroleukemic cells

and colon cancer (Cox 1985; Cox and Goorha 1986; Fiala et al. 1998). All these studies have primarily focussed on DNMT enzyme activity using high concentrations of selenium. For instance, Fiala et al. reported that p-XSC showed a dose-dependent inhibitory effect of DNMT activity with an IC_{50} of 20 µM in HCT116 cells. In contrast, nutritionally-relevant doses of different forms of selenium and/or isothiocyanates were employed here. Although the study conducted by Fiala et al. has showed a reduction in the level of DNMT1 by p-XSC, recently it has been reported that targeted disruption of DNMT1 alleles in HCT116 and in other human colon cancer cells did not disrupt aberrant CpG island hypermethylation status (Ting et al. 2004). This important finding implies that targeting the DNMT1 enzyme alone may be insufficient to re-activate epigenetically silenced tumour-suppressor genes and restore normal cellular growth in colorectal cancer cells due to the compensatory functions of other DNA methyltransferases in establishing and maintaining epigenetic gene silencing (Jones and Liang 2009). In support of this theory, Figure 3.8 shows that although Caco-2 cells treated with SFN for 4 days caused a statistically significant downregulation of DNMT3A and DNMT3B mRNA, there was no effect on DNMT1 expression, suggesting that the downregulation of these two enzymes by SFN was not sufficient to modify the methylation status of the CpG islands studied (Figure 3.6 and 3.7).

In relation to the epigenetic effects of isothiocyanates, a study has revealed the importance of cruciferous vegetable consumption in relation to DNA methylation in primary gastric carcinomas. This study analysed the methylation status of the caudal type homeobox transcription factor-2 gene (*Cdx2*), a gene known to be positively correlated with the development of gastric cancer when its expression is lost. After comparing the level of methylation with the past lifestyle of the patients, including dietary habits, an increased intake of cruciferous vegetables was significantly associated with decreased frequency of *Cdx2* methylation in male patients (Yuasa et al. 2005). There are no direct human studies available on the effect of food compounds on the epigenome. However, there is one pilot human study reporting changes in HDAC activity

in healthy volunteers who consumed single doses of SFN-rich broccoli sprouts (68 g, one cup), in which blood was drawn after sprout consumption, showing that HDAC activity was inhibited in peripheral blood mononuclear cells (Myzak et al. 2007).

On the other hand, studies carried out in a human myeloma cell line have found that phenylhexyl isothiocyanate (PHI) induced histone H3 hyperacetylation and *p16* hypomethylation in a concentration dependent manner, suggesting that PHI has dual epigenetic modulating effects on both DNA methylation and chromatin (Lu et al. 2008). However, the authors concluded that, additional to the effect of this ITC on HDAC inhibition, the demethylation of *p16* might involve an effect of PHI on DNA methyltransferase. Comparable results were obtained in prostate cancer cell lines, where PEITC inhibited the activity and level of histone deacetylases and promoted *GSTP1* promoter demethylation (dual action) (Wang et al. 2007), but this study similarly did not elucidate if this ITC acted directly on the DNA methyltransferase enzyme. Another study identified a 1.8-fold downregulation of DNMT1 after conducting a microarray analysis of human Caco-2 cells exposed to SFN (Traka et al. 2005). However, the concentrations of SFN used in this study ranged between 25-50 µM, which as shown in section 2.2.1 are toxic and promote a decrease in cell viability.

More recently the involvement of isothiocyanates on DNMTs has recently been confirmed in a study in which sulforaphane was found to inhibit DNMTs in breast cancer cells. Meeran et al. (2010) showed that sulforaphane treatment dose- and time-dependently inhibited human telomerase reverse transcriptase (hTERT), the catalytic regulatory subunit of telomerase, in both MCF-7 and MDA-MB-231 human breast cancer cells and that it had insignificant effects on normal control cells. Furthermore, DNMT protein expression (particularly DNMT1 and DNMT3A), was also reduced in SFN-treated breast cancer cells. Additionally, site-specific CpG demethylation was observed primarily in the first exon of the h*TERT* gene. This facilitated binding of CCCTC-binding

factor (CTCF), which is associated with h*TERT* repression, leading to cellular apoptosis of the breast cancer cells (Meeran et al. 2010).

Imbalances of nutrients have been shown to lead to global DNA hypomethylation. Dietary methyl deficiency of folate, choline, and methionine has been shown to reduce the global methylation status of the genome (Duthie et al. 2000; Wasson et al. 2006). A depletion in selenium has also been shown to cause a similar effect, as shown by the induced global hypomethylation in Caco-2 or HT-29 cells lines and in rat liver and colon (Davis et al. 2000; Davis and Uthus 2002). In this respect, genome-wide DNA hypomethylation has been shown to play an important role in genomic instability by reactivating transposable DNA sequences during colorectal carcinogenesis (Ogino et al. 2008). However, the results obtained for Caco-2 and HCT116 cells showed the 5-methylcytosine content in the genome was unchanged following selenium and/or isothiocyanate treatment. There was a difference in methodology for quantifying global methylation between this study, which measured LINE-1 methylation, and the previous studies by Davis et al (2000) and others, which used the *in vitro* methyl acceptance method. This uses tritium-labelled S-adenosylmethionine [^{3}H-methyl] as a methyl donor in the presence of the M.SssI DNA methyltransferase, so that the number of radiolabeled methyl groups incorporated into DNA is inversely proportional to DNA methylation status. Additionally, apart from differences due to specific DNA methylation measurement techniques employed, it is also likely that other factors affecting the DNA methylation status of the cell such as: cell passage number, cell confluence by the time of harvesting, treatment exposure time and cell type may have influenced the results obtained and their comparability. However, several controls were included in this investigation that validated the results obtained.

In conclusion, dysregulation of methylation patterns is a common characteristic in tumour cells observed in almost all types of cancer. Several previous studies suggest that diet-derived factors offer great potential for the prevention and

therapy of a wide variety of cancers by altering various epigenetic modifications. However, the literature and our results suggest that, while some tissues may respond effectively to particular food compounds by impacting levels of gene-specific methylation, others do not respond. Also, even within the same tissue some promoter region CpG islands are affected but others remain unaltered. Although for some genes it has been suggested that methylation occurs in an 'all-or-none' manner (Belshaw et al. 2005), it is also apparent that particular cytosines within a CpG island can have a distinct likelihood of being methylated (Mund et al. 2005). This raises the question of whether there are regions of the epigenome that are more susceptible than others to dietary constituents.

This study has consistently shown that treatment of colon cancer cells with selenium and/or isothiocyanates, either individually or in combination, does not impact abnormal methylation patterns of key genes involved in the complex multistep process of colon carcinogenesis. Therefore, more research is warranted in this area to determine the ability of these food compounds to alter the complete epigenetic setting of the transformed cell, including histone modifications and DNA methylation, chromatin remodelling factors and CpG binding proteins, which have been shown to work in concert to establish DNA methylation patterns in the colorectal genome.

4

Applications and Challenges of Genome-wide DNA Methylation Analysis using CpG Island Microarrays in Nutrition Studies

4.0 INTRODUCTION

As shown in the previous chapter, targeted DNA methylation assays can provide important insight into the effects of dietary compounds on the methylation status of designated CpG islands. However, cancer is a disease of the genome and as a result generates an accumulation of genetic and epigenetic disorders altering the balance between cell proliferation and apoptosis, affecting several types of genes including oncogenes and tumour suppressor genes. Before the advent of 'OMIC' technologies, studies were limited to the interpretation of changes in DNA methylation only by analysis of total 5-methylcytosine content and by examination of selected small regions of DNA (usually in or near the promoter region of genes) chosen on the basis of probable relevance to tumour development.

Currently, a more efficient way of linking the different pathways involved in cancer epigenetics and nutrition at the genome-wide scale involves the use of CpG island microarrays to allow the determination of the methylation levels of a large number of CpG island loci, potentially modified as a result of the action of bioactive food compounds. To increase our understanding of the epigenetic mechanisms of colon cancer chemoprevention by Se-methylselenocysteine, iberin and their action in combination we attempted to conduct a novel CpG island microarray analysis to elucidate their effects on the epigenome of Caco-2 cells.

The initial focal point of this chapter was to determine the impact of these bioactive food constituents upon the epigenome through the use of genome-wide CpG island methylation analyses together with pioneering statistical models to provide clues for understanding their chemoprotective effect in colon cancer and potentially detect novel genes that are regulated through DNA methylation marks. As a result of the findings, however, we tackle the imperative need to continue the search for new statistical models and

microarray platforms to assist in the optimal estimation of methylation changes in CpG island microarray studies. Such models are particularly important in the field of nutrition, in which it is expected that many dietary bioactive agents at nutritionally-relevant concentrations will probably elicit subtle DNA methylation changes that may be critically important in biological terms but will be difficult to detect reliably.

This chapter is structured as follows. In the first section an introduction to the initial microarray protocol applied to interrogate the methylome is outlined, followed by a description of an additional microarray approach adopted in order to dissect causes accountable for the lack of correlation observed between the data generated by the CpG island microarray platform and genes selected for validation. Finally the results and conclusions generated from this investigation will be presented.

4.1 MATERIALS AND METHODS

4.1.1 Materials

CpG island microarrays (HCGI12K-Human) consisting of 12192 CpG Island clones were purchased from the Microarray Centre, University Health Network (UHN) in Toronto. For the microarray set-up and validation random primers, dNTP and Human Cot-1 DNA were purchased from Invitrogen, UK. Klenow Fragment (3'→5' exo), McrBC and all the different methylation-sensitive restriction enzymes used for the microarray validation were obtained from New England Biolabs, UK. Illustra^{TM} CyScribe GFX purification kit, CyDyes (Cy3 and Cy5) and HyPer5 Dye were acquired from GE healthcare. tRNA from baker's Yeast, Aminoallyl-dUTP and all other chemicals were purchased from Sigma-Aldrich (UK), unless otherwise stated.

4.1.2 Methods

4.1.2.1 Cell Culture and Treatments

Caco-2 cells and treatment protocols used for this investigation correspond to those specified in Chapter 3 (section 3.1.2.1).

4.1.2.2 First Microarray Protocol

A schematic diagram containing all the steps involved for the investigation of differentially methylated CpG islands using microarray-based DNA methylation profiling is presented as Figure 4.1.

4.1.2.2.1 Amplicon Preparation

4.1.2.2.1.1 *Mse*I Restriction

Genomic DNA from two biological replicates per treatment was isolated from Caco-2 cells treated for 4 days with either SeMSC (0.2 µM), iberin (6 µM) or in combination (IB+SeMSC) according to the protocol specified in chapter 3 (section 3.1.2.2). Two biological replicates per treatment were included for this experiment. The genomic DNA (500 ng) was digested with a four-base frequent cutter *Mse*I [thymine/thymine/adenine/adenine (5'-TTAA)], which produces small fragments (≤200 bp) and generally cuts outside of CpG islands. The digestion mixture included 5 µl 10×NE buffer 2, 0.5 µl bovine serum albumin (BSA, 10 mg/ml), 1 µl *Mse*I (10 U/µl) and water to 50 µl. The digestion was allowed to proceed overnight at 37ºC and the digested product was then purified using a slight modification of the QIAquick® PCR purification kit instruction manual. Briefly, 5 volumes of buffer PBI were added to 1 volume of the sample and the mixture was placed into the QIAquick column and centrifuged for 60 sec. To wash, 0.75 ml of buffer PE were added twice to the column and centrifuged for 60 sec each time. The flow-through was discarded and centrifuged for an additional 5 minutes and then the DNA was eluted by adding 100 µl of buffer EB to the centre of the QIAquick column, which was then centrifuged for 1 min. After this step, DNA was precipitated by adding one-tenth volume of 3M NaOAc, pH 5.2 to the nucleic acid solution followed by two and a half volumes of 95% cold absolute ethanol; this solution was placed at -80°C for at least 30 min, or at -20°C overnight, and centrifuged at 4ºC for 30 min to pellet the DNA. Supernatant was discarded carefully with a pipette and the pellet was washed with 200 µl 70% ethanol and spun for 5 min, dried and resuspended in 10 µl QIAquick elution buffer and stored at -20ºC.

4.1.2.2.1.2 Annealing of Oligonucleotides

2 nmol of each oligonucleotide that formed the linker (upper strand sequence, 5'-TAGAATTCAGATCTCCCG-3'; lower strand sequence, 3'-CTTAAGTCTAGAGGGCCCAGTGGCG-5') were mixed with 30 µl of Tris-EDTA buffer (10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA) and placed in a thermal cycler (Biometra T-Gradient, UK) to complete the following cycles: I) heating to 96ºC for 5 min. II) ramp cool to 85ºC for 5 min and gradually cool down by 1ºC for 5 min until 75ºC is reached. III) ramp cool to 74 for 15 min and gradually cooled in 1ºC steps for 15 min until 55ºC is reached (Table 4.1).

Table 4.1 Thermocycler Programs for Annealing Complementary Oligonucleotides

	Cycles	Temperature	Time
Step 1 :	1	96 ºC	5 min
Step 2 :	1	85 ºC	5 min
Step 3 :	10	85 ºC (-1ºC/cycle)	5 min
Step 4 :	1	74 ºC	15 min
Step 5 :	19	74 ºC (-1ºC/cycle) until it reaches 55 ºC	15 min

4.1.2.2.1.3 Linker Ligation to *Mse*I-Digested DNA

The annealed linker primers (6 µl/sample) were added to 10 µl of *Mse*I-digested DNA to allow subsequent amplification of all fragments in the ligated DNA samples. The reaction was carried out in the presence of 2.5 µl of T4 ligase (1 U/µl) and 1.5 µl of 10×ligase buffer. The ligation reaction was completed by incubating the samples overnight at 16ºC in a thermal cycler (Biometra T-Gradient, UK). The linker-ligated DNA was then purified using the QIAquick modified protocol, eluting into 30 µl of EB.

4.1.2.2.1.4 Digestion of Linker-Ligated DNA ± McrBC

To proceed with the McrBC digestion an aliquot of 14 µl of the linker- ligated DNA was added to 6 µl of +McrBC mix and an additional aliquot was added to 6 µl of –McrBC mix. The +McrBC mix contained 2 µl 10×NE buffer 2, 2 µl McrBC (20 units), 0.2 µl bovine serum albumin (BSA, 10 mg/ml), 0.2 µl GTP (100 mM) and water to 6 µl, whereas the –McrBC mix contained the same except McrBC. To determine the efficiency of the digestion a methylated DNA control (New England Biolabs, UK) was included by adding to each mix (+McrBC and –McrBC) 1 µl control DNA and water to 20 µl. The linker-ligated DNA was then digested overnight at 37°C. Twenty microliter of each digested control was analyzed on a 1% agarose gel. A diffuse smear between 2500 and 1500 bp indicated a successful McrBC digestion. The digested samples were QIAquick purified and ethanol precipitated as above and resuspended in 10 µl of EB for PCR amplification. McrBC is a methylation-specific endonuclease, which cleaves DNA containing 5-methylcytosine on one or both strands but will not act on unmethylated DNA. The low-specificity of the recognition site for McrBC ($R^{me}C..N_{40\text{-}3000}..R^{me}C$, in which R is A or G) suggests that McrBC-based analysis can reveal DNA methylation at almost any methylated CpG island in the genome. The digested and mock-digested DNA was then used as template for PCR amplification, using the lower strand sequence linker-primer. Cleavage of methylated DNA by McrBC induced DNA strand breaks and abrogated PCR amplification. Conversely, the presence of unmethylated cytosines in DNA prevented enzyme cleavage and could be detected by PCR amplification. Each PCR reaction contained 20 ng of linker ligated DNA ± McrBC, 50 pmol of primer, 200 µM each dNTP, 2 µl 10×PCR reaction buffer with $MgCl_2$ (Roche), 0.2 µl BSA and 0.2 µl Taq (5 U/µl) (Roche) in a total volume of 20 µl. The cycling conditions consisted of an initial 5 min at 72ºC to fill in the protruding ends of the ligated DNA, followed by 22 cycles of 1 min at 95°C and 1 min at 60°C, ending with an extension at 72°C for 1 min. The final PCR product was QIAquick purified into 50 µl EB.

4.1.2.2.2 Labelling and Hybridization

4.1.2.2.2.1 Aminoallyl-dUTP Incorporation

For labelling and hybridization the methodology established by the Microarray Centre at UHN was followed. Briefly, 4 µg of purified PCR-amplified, digested linker-ligated DNA ± McrBC was mixed with 20µl of 2.5X random primer buffer (125 mM Tris–HCl, pH 6.8; 12.5 mM $MgCl_2$; 25 mM 2-mercaptoethanol; 1250 µg/ml random primers) and Arabidopsis DNA (10ng/µl), which was used as a control for signal normalization, in a final volume of 40 µl. The mixture was denatured at 95ºC for 5 min. The denatured sample was placed on ice for 10 min and 5 µl of NEB buffer 2, 5 µl of aminoallyl-dUTP-dNTP mix (containing 5 mM of each dinucleotide, except dTTP 1 mM and 4 mM of aminoallyl-dUTP) and 1.5 µl of high-concentration Klenow fragment (50 U/µl) were added. The sample was incubated overnight at 37ºC. The resulting product was purified using the CyScribe GFX purification kit according to manufacturer's directions. Briefly, 50 µl RNase-free water was added to each sample and mixed with 500 µl of capture buffer by pipetting up and down and the sample was transferred onto a GFX column. Samples were centrifuged at 13,800 x *g* for 30 sec, discarding the flow through. Following this step, 600 µl of 80% ethanol were added to the column, which was centrifuged for 30s at 13,800 × *g*, repeating this step twice for a total of three washes. The column was transferred to a new 1.5 ml tube to elute with 60 µl of pre-warmed (65ºC) RNase-free water followed by an incubation step of 10 min. Samples were centrifuged for 1 minute at 13,800 × *g* and 60 µl of $NaHCO_3$ pH 9.0 were added on the top of the column to increase the yield, centrifuging again for an additional 1 minute. DNA was dried down in a SpeedyVac at medium setting and re-suspended in 8 µl of RNase-free water.

4.1.2.2.2.2 Cy Dye Coupling

Cy3 and Cy5 dye packs were obtained from GE Healthcare. As these dyes are very sensitive to light and moisture, each dye pack containing 40.000 pmol/vial was reconstituted in 16 µl of DMSO and equally aliquoted into 4 microtubes. The DMSO was removed subsequently by drying in a SpeedyVac and reagents were then stored at -20°C in the presence of silica. To prepare the coupling reaction an aliquot of each dye was re-suspended in 2 µl of DMSO and added to each aminoallyl-labeled sample. For instance, the + McrBC was mixed with Cy5 dye and the – McrBC mixed with Cy3 dye, followed by an incubation step at room temperature for 2 hours in the dark. After this step, the two fractions were combined in a tube and 45 µl of water was added to the sample and gently mixed. The uncoupled Cy dye was removed by using the CyScribe GFX purification kit (as described above), but eluted with 60 µl of 65°C pre-warmed elution solution. Finally, 5 µl of CotI DNA (1 µg/µl) was added per sample and samples were dried in a SpeedyVac at high setting to 2.5 µl.

4.1.2.2.3 Microarray Hybridization

Samples were added to 80 µl of a hybridization mixture containing 80 µl DIG EasyHyb (Roche) and 4 µl Yeast tRNA (10mg/ml). Prior to adding to the samples the mixture was denatured at 65ºC for 3 minutes and cooled at room temperature for at least 15 min. After adding 80µl of this master mix to each labelled target the mixture was denatured further at 65ºC for 2 minutes and cooled briefly before being applied to the microarray slide. To prepare the hybridization slides, the microarray slide was placed face to face with a microscope slide to act as ‘coverslip’ and left offset by about 2 mm along their length. The entire labelled cDNA mixture was pipetted onto the resulting ledge and once the solution had spread through capillary action the slides were reset to be completely aligned and were placed in a humidified hybridization chamber and incubated overnight at 37°C.

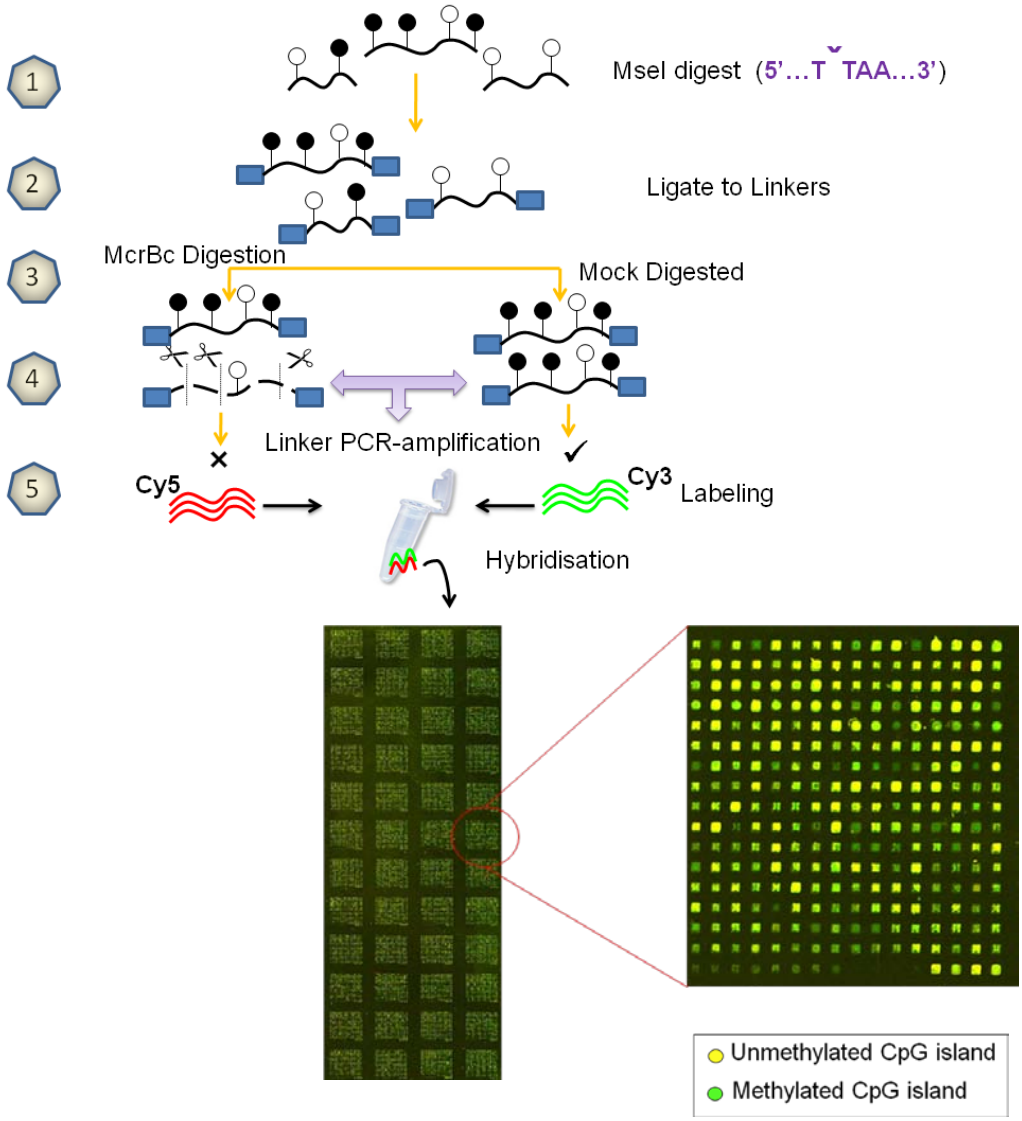


Figure 4.1 Schematic flowchart for the preparation of amplicon:. 1) Genomic DNA was digested with *Mse*I. 2) The digested DNA was ligated to oligo linkers. 3) The linker ligated DNA samples were divided in two: one sample was digested with a methyl-specific restriction enzyme (McrBC), and the other was mock digested. 4) Each sample was PCR amplified using linker primers. Cleavage of methylated DNA by McrBC induced DNA strand breaks and abrogated PCR amplification. Conversely, the presence of unmethylated cytosines in DNA prevented enzyme cleavage and could be detected by PCR amplification. 5) Samples were labelled and hybridized to the microarray slides.

4.1.2.2.4 Washing and Scanning

The microarray slide was inverted in 1 × SSC to separate from the 'coverslip'. Each microarray slide was placed in a slide rack containing also 1 × SSC. Once all the cover slips were removed the slides were washed three times for 15 minutes each in 1 × SSC and 0.1% SDS solution at 50ºC, agitating the slides half way through each step. The slides were then rinsed with 1 × SSC at room temperature (by moving the slides back and forth about four times) to remove all traces of SDS. After this step, an additional quick rinse was performed at room temperature using 0.1 × SSC. The slides were dried by centrifuging at 500 rpm for 5 minutes and scanned immediately with the AGILENTGenePix Pro 6.0 scanner with a scan resolution of 5-microns and a photomultiplier tube (PMT) saturation set to 100% for the green and red channel. The red and green channel images were separated into TIFF files using the TIFF splitter utility from the software.

4.1.2.2.5 Data Processing

After obtaining the images they were analysed in the GenePix Pro 6.0 software. Each spot was defined by the positioning of a grid template (obtained from UHN Toronto website) over the array image. Within the array images different fluorescent signals (colours) were obtained according to the initial methylation status (Figure 4.1). For instance, if within the McrBC digested sample (labelled with Cy5) a gene was methylated the locus would fail PCR amplification as a result of the enzyme digestion that cleaved the methyl-cytosine regions, failing to produce a labelled target for microarray hybridization and generating as a result a Cy3/Cy5 ratio (undigested/digested) greater than one, which would produce a green feature. Likewise, if methylation was absent the enzyme would not act upon the strand and the signal intensities of Cy5 and Cy3 would be equivalent, generating as a result a yellow spot.

4.1.2.2.6 Data Normalization

All the analysis of the data and normalization was carried out in collaboration with the University of Maastricht in the department of Bioinformatics, BiGCaT by Michiel Adriaens. Briefly, quintile normalization was used on the signal intensity values. The log-ratio of the Cy3 and Cy5 channels were used as a measure for methylation and the differences in log-ratio between the treatment groups and the control arrays were used as a measure for differential methylation. For instance:

1. Selenium 1 vs. Control 1
2. Selenium 2 vs. Control 2
3. Selenium 1 vs. Control 2
4. Selenium 2 vs. Control 1

For statistical significance a one-way ANOVA was used to calculate p-values. To avoid falsely-significant results the Benjamini-Hochberg False Discovery Rate (FDR) test for multiple comparisons was applied, using a p-value cut-off of 0.05. This process leads to all genes with an FDR-adjusted value of less than 0.05 being considered as differentially methylated (Curtis et al. 2005). In addition, probes were filtered on probe length (>25 bp), probe quality (verified sequence) and proximity of the probes to genes (i.e within 2000 bp) before all analysis procedures were carried out.

A value of 0% methylation was used when the red signal equalled the green signal and 100% methylation if the red signal was equal to zero.

4.1.2.2.7 Validation of Detected Methylation Differences

A modified combined bisulphite restriction analysis (COBRA) assay (Xiong and Laird 1997), was initially chosen to validate several loci that displayed statistically significant differences in methylation. This method is based on the restriction digestion of a PCR product amplified from bisulphite-modified DNA with an enzyme for which the recognition sequence is affected by the methylation state in the original DNA. Additionally, a second method that involves the digestion of genomic DNA using methylation-sensitive restriction enzymes followed by real-time PCR was adopted.

4.1.2.2.7.1 COBRA

4.1.2.2.7.1.1 COBRA PCR Primer Design

To design the primers for the genes selected for validation the NCBI website (Entrez Gene) was used to identify the CpG island sequence spotted onto the microarray slide (provided by the UHN Microarray Centre's CpG Island Database) using the sequence viewer in the NCBI website. After this, as the library for the microarray was generated by the manufacturer by cutting the genomic DNA with *Mse*I, the sequence was transferred onto Webcutter version 2.0, (http://users.unimi.it/~camelot/tools/cut2.html) to map the *Mse*I restriction sites within the sequence and identify the CGI array probe. Likewise, the Webcutter website was used to identify potential recognition sites for restriction enzymes commonly used in the COBRA assay (containing cytosines only in the context of CpG). It is important to mention that the restriction-enzyme cleavage itself is not methylation-dependent as PCR products from bisulphite converted DNA do not contain 5-methylcytosine. The methylation status is revealed by the presence or absence of a restriction-enzyme site.

Once the gene sequence and their accompanying restriction maps were generated, PCR primers were designed for bisulphite treated genomic DNA. The bisulphite-converted methylated sequence was used to facilitate the avoidance of CpG dinucleotides within the primer sequences, ensuring equal annealing and therefore amplification of DNA regardless of original methylation status.

4.1.2.2.7.1.2 PCR Annealing Temperature Optimization

To determine optimal annealing temperatures of the primers a thermal cycler (Biometra T-Gradient, UK) that generates a thermal gradient of up to 12 different temperatures was used. Each PCR reaction was prepared according to the details provided in section 3.1.2.4 (Chapter 3) and then all the reactions were electrophoresed on a 4% agarose gel electrophoresis to identify the annealing temperature that produced a single bright band.

4.1.2.2.7.1.3 Outline of the COBRA Protocol

Genomic DNA isolated from the experimental samples was initially bisulphite converted following the steps outlined in Chapter 3 (section 3.1.2.3.1). In order to interrogate the methylation status of the CpG island at the locus specified in Table 4.2 from the 10 genes selected for validation, products from the primary PCR were precipitated with ethanol followed by an overnight digestion at the recommended temperature. Reactions contained the purified PCR product, 5 µl 10× NE enzyme buffer, 2 µl of the chosen restriction enzyme (Table 4.2), 0.5 µl BSA (if required) and water to 10 µl. The digested DNA was separated on 8% (product size <350 bp) or 6% (product size > 350 bp) polyacrylamide gels in TBE. After electrophoresis, the gels were stained using SYBR green I and imaged using the Pharos FX+ Imaging System (Bio-Rad, UK) at 100-µm resolution; images were saved as TIFF files and band intensities were quantified using TotalLab 2D analysis software (Nonlinear Dynamics,

Newcastle-upon-Tyne, United Kingdom). The proportion of methylated versus unmethylated DNA was determined from the relative intensities of cut and uncut PCR product.

Table 4.2 Primer Sequences and Annealing Temperatures for the Analysis of Methylation at the Indicated CpG islands

Gene	PCR	Primer Sequence (5' – 3')	Ann Temp optimized	Product Size (bp)	Restriction Enzyme (Cuts at)*
COL1A1	F R	GTTTTGAGATAGGAGGGAGTTTA AATCTTTCCTTATAAATCATCCCA	57.6	257	Mae II (158)
DUSP10	F R	GGTTTTATTGATTTTTAGTAGTAATATAGTTAT CCTCCTTAAAAAAATAAAACCA	55	223	Hinf I (77)
PDCD10	F R	GGGGATTGGGATAGAATAGTTAT TCTCCTACTATTAACTAAAAATATCACCA	58.8	300	Aci I (253)
TIRAP	F R	GTTTGGGTTTTAGAGTTT AAAAAAATAACATTCTCCTC	51.6	396	BstUI (46)
PIK4CB	F R	GAAGTATGGATATATGTATATTTTTAGAAGTA TCACAAACTACCCAAACAAA	54	382	Sau3AI (58)
EPHA7	F R	GTGATGTAGTTATTTATAGTTTAAATTTTAG CCTATATAAATACTAAACCCATTCA	54	279	Sau3AI (138)
IL4R	F R	TTAAAGTTAGTTTGGGTGTTGTAAT CCCCTAACACTTTAACTAAACAAA	57	275	Hinf I (99)
SIRT2	F R	GGGTTTGTAGTATTTAGTTAGGTT CCCTTTACCAACATAACTACTAA	58	468	Pvu I (116)
DISHEVELLED-3	F R	TTTTGGTTTTTAAGGGATGA CTAAAACCAAAAAAACCCAA	56	112	Taq I (32)
MYST1	F R	GAGGTTGAGGTTGGTAGA CCCAAACTAAAATACAAAAA	54.8	215	EcoR I (158)

*CpG locus interrogated by the restriction enzyme

4.1.2.2.7.2 Methylation-Sensitive Restriction Enzymes (MSRE)

At present there are a variety of methods available for the determination of methylation patterns and the quantitative assessment of gene-specific methylation in sample tissues. These include the previously mentioned COBRA technique for interrogating methylation status of individual CpG sites present in a gene and several others as mentioned in Chapter 3 (section 3.0.1).

Currently, a large number of available techniques used for studying genome-wide methylation differences involve the use of methylation-sensitive restriction enzyme(s) (MSRE) thereby limiting these approaches to profile genomic regions containing these restriction site motifs. Among these methods, an improved quantification of DNA methylation using methylation-sensitive restriction enzymes and Real-Time PCR was reported (Hashimoto et al. 2007) and was adopted in this investigation as an alternative approach to confirm the methylation status of one or more CpG islands within the genes chosen for validation. The MSRE cleaves DNA when the CpG site is not methylated and subsequent PCR amplification is abrogated. However, if the CpG site is methylated, the enzyme cannot cut and the DNA strand is amplified by PCR.

4.1.2.2.7.2.1 PCR Primer Design for Real-Time PCR Following MSRE Digestion of Genomic DNA

Unlike the COBRA technique mentioned above, this methodology avoids the bisulphite treatment which is cumbersome, labour-intensive and might generate incomplete conversion and loss of DNA in the sample. In order to design the primers for the genes chosen for validation and to identify the number of restriction sites cleaved by the selected methylation-sensitive restriction enzyme used in this study: *Hpa*II (C$^{\downarrow}$CGG), similar steps to those described in section 4.1.2.3.1.1 were followed. However, as this methodology does not rely on bisulphite conversion of genomic DNA, primers were designed (Table 4.3) according to the standard principles for successful quantitative PCR and their annealing temperature was similarly optimized as described in section 4.1.2.3.1.2.

Table 4.3 Primer Sequences and Annealing Temperatures used for Methylation-sensitive RT-PCR

Gene	PCR	Primer Sequence (5' – 3')	Ann Temp optimized	Product Size (bp)	Number of Restriction sites the Enzyme HpaII Cuts at
VASP	F R	CGCTCCGCACACAATCTGCTT TCCAGCTCCCCCTCCTCCA	65.9	310	4
CTNND2	F R	TGGGAAAAGTCTCTTCTGGA ATAAGCACTCAGAACCTTCCAG	57.5	73	1
CBX5	F R	GGGTAGATAAGACTGTCTGCCA GTAGTGGGCGGAGAAAAAA	57.5	204	3
PIK4CB	F R	GATGGACGCTGGGTTCCTAGAA GCAGGGAAGATGGCATTCAA	65	728	2
CHD2	F R	TCAGAAATTAAGAATTCAGAAAAGT AAAGTGACCCTAGCTATGTCTAACT	57.5	382	2
FUS	F R	CTCGTGTTGGTTCAGCTTTCTGT GAACAAGATCAACTCATCCTCCCA	65	686	2
DKFZP564O0823	F R	ACTGCAGGTAATTGGCGCCAT ACGATTGCGAGCCTGACCA	65	592	6
GALNT7	F R	CACTGTAGGACATGATTCAAGA TGCTTACTAACATCATACCCATCTT	57.5	750	1
ATOH	F R	TGTCCTCTGGGAACATAGAA TTTCCGCTTGAGCTTCTTA	59	641	2

4.1.2.2.7.2.2 Outline of the MSRE Protocol

To digest the genomic DNA with *Hpa*II the reaction mixture contained 2 units of *Hpa*II per 20 ng of genomic DNA, 1 µl 10 × NE enzyme buffer and water to 10 µl. The non-enzyme control or mock-digested reaction was identical except *Hpa*II was not added. All prepared samples were incubated overnight at 37°C followed by heat inactivation at 65°C for 20 minutes. The proportion of DNA/*Hpa*II employed here has been previously reported to be optimal by Hashimoto and colleagues (2007). This author has recommended using more than 10 ng/reaction to avoid false negatives and to use low restriction enzyme concentrations (2 units) to prevent non-specific digestion.

The Real-Time PCR was performed on the digested and mock-digested samples to determine the methylation status as a percentage. Reactions were mixed in a total volume of 20 µl with 5 µl of template DNA. The remaining components and conditions used to run the plate correspond to those described in Chapter 3 (section 3.1.2.5). The optimized annealing temperature used for every gene analysed is presented in Table 4.3 Serial dilutions of DNA of known concentration (250-0.05ng) were run in every reaction plate to produce standard curves for the determination of the DNA content of each sample. Changes in the DNA content of the digested samples were expressed relative to the mock-digested sample and multiplied by 100 to determine the percentage of methylation. As a quality control procedure DNA was artificially methylated according to the steps described in Chapter 3 (section 3.1.2.8.1) and included in every reaction to estimate nonspecific digestion. Initially, optimization experiments were conducted with this fully methylated DNA to determine to what degree actual measurements differed from the expected results and the results obtained were close to 100% indicating a high degree of accuracy for this analytical tool.

4.1.2.3 Second Microarray Protocol

A lack of correlation between the results of the microarray analysis and the validation process was observed as will be seen in the results section. A second microarray set-up was therefore performed as described in the sections above but with adaptations taken from another study (Ordway et al. 2006) to tackle potential drawbacks in the previously described microarray protocol. These introduced changes were limited to the in-house protocol for amplicon preparation detailed in section 4.1.2.2.1, retaining the original steps as described in sections from 4.1.2.2.2 and onwards.

4.1.2.3.1 Sample Preparation

In order to determine the sensitivity of the chosen microarray-based methylation analysis platforms a mixture of fully methylated and non-methylated DNA was prepared to contain different ratios of methylated DNA fragments: 0%, 30%, 60% and 100%. The DNA source from the non-methylated sample used from which the 0% fraction was derived, was the normal colon tissue biopsy of a healthy young person described in Chapter 3, which was artificially methylated (section 3.1.2.8.1) to create the fully methylated fraction (100%). Every fraction contained 15 µg of DNA in 300 µl of water (Table 4.4).

Table 4.4 Methylated and non-methylated DNA fractions used to generate specific DNA methylation profiles

Set DNA Methylation Profile (%)	Fully Methylated DNA fraction (µg)	Non-Methylated DNA fraction (µg)
100	15	0
60	9	6
30	4.5	10.5
0	0	15

4.1.2.3.2 Amplicon Preparation

4.1.2.3.2.1 DNA Fractionation

Colon DNA obtained from the human tissue sample was mechanically sheared to a uniform molecular weight distribution using a HydroShear® DNA shearing device (GeneMachines, Washington USA) (Figure 4.2). The principle of this system relies on hydrodynamic shearing forces that stretch the DNA

until its molecular bonds begin to break and the DNA snaps into fragments (Oefner et al. 1996).

Figure 4.2 HydroShear DNA shearing device used to fractionate the DNA into smaller fragments.

As the DNA fragment size required corresponded to approximately 2 kb, different settings of the HydroShear DNA device were optimized first to obtain the desired length. To achieve this, DNA isolated from Caco-2 cells was run at various Speed Code values and Number of Cycles (Table 4.5). The sheared samples were then run on an agarose gel to verify fragment sizes (Figure 4.3).

Table 4.5 Settings applied to the HydroShear machine to optimize DNA fragment size (see corresponding Figure 4.3)

Gel Lane	*Number of Cycles*	*Speed Code*
2	10	5
3	10	6
4	10	7
5	10	8
6	10	9
7	10	10
8	10	15
9	15	8
10	20	8
11	20	8
1, 12	Hyperlader I	

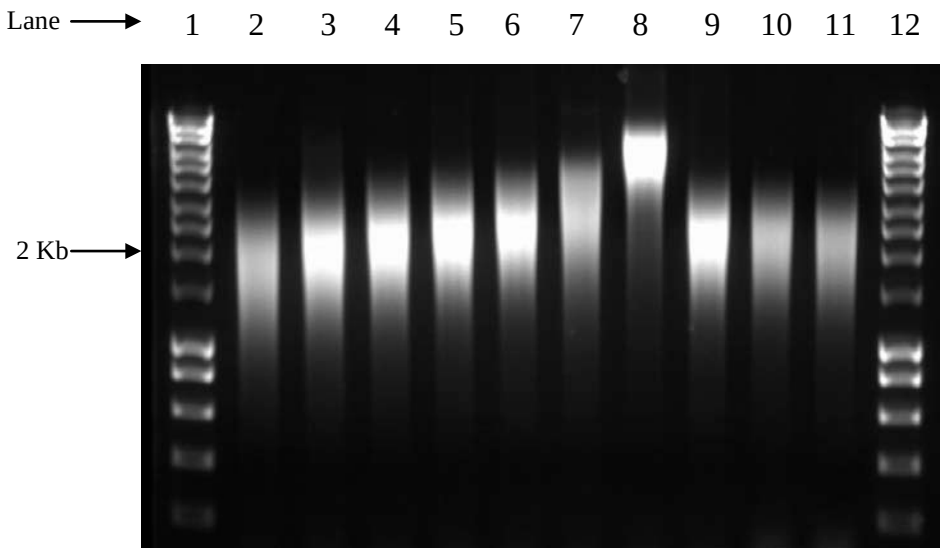


Figure 4.3 The HydroShear DNA shearing device generates different fragment sizes depending on the setting applied (see Table 4.5). The figure corresponds to a 1% agarose gel in 1 × TAE stained wtith Ethidium Bromide, run at 95V for 1 hour. 1µg of DNA sample was loaded per lane in a final volume of 20 µl containing loading buffer.

From these results the settings that were selected to be applied to the samples with different DNA methylation profiles (Table 4.4) correspond to those shown in Lane 3 (Number of Cycles =10; and Speed Code=6). Three separate biological replicates per DNA fraction were used.

4.1.2.3.2.2 McrBC Digestion

After DNA samples were mechanically sheared into a uniform molecular weight distribution samples were measured using a NanoDrop (Labtech International, UK). For each sheared genomic DNA sample 5 µg were obtained and split into two equal portions of 2.5 µg each. One portion was digested with McrBC in a reaction containing 10µl 10×NE buffer 2, 5 µl McrBC (50 units), 1 µl bovine serum albumin (BSA, 10 mg/ml), 1 µl GTP (100mM) and water to 33 µl. The remaining portion was mock-digested under identical conditions except that 5 µl water was added instead of McrBC. Treated and mock-treated reactions were incubated at 37ºC overnight and digestion was confirmed by running the control DNA sample supplied with the McrBC enzyme kit on a 1%

agarose gel (data not shown). All reactions were treated with 5 µl proteinase K (50 mg/ml) for 1 h at 50ºC, and precipitated with ethanol under standard conditions. Pellets were washed twice with 200 µl ethanol, dried and resuspended in 20 µl water. A total of 2.5 µg of each fraction was resolved in a 1% agarose gel. McrBC-treated samples and mock-treated samples were resolved side-by-side. HyperLadder I was run adjacent to each treated/untreated pair to guide accurate gel slice excision. Gels were visualized with long-wave ultraviolet (UV), and gel slices including DNA within the modal size range of the untreated fraction (~1-4 kb) were excised with a clean razor blade (Figure 4.4). DNA was extracted from gel slices using the QIAquick Gel extraction kit protocol (Qiagen, UK). Then, samples were analyzed for total concentration using the NanoDrop. A total of 300 ng of fractionated template DNA for dye labelling and microarray hybridization was used following steps described in section 4.1.2.2.2. Since the signal intensity generated from the Cy5 dye during the first microarray carried out was noticed to be lower than that generated by the Cy3 dye and considering that others have also detected that Cy5 is highly susceptible to ozone-induced degradation (Branham et al. 2007), HyPer5 from GE Healthcare, a red fluorescent dye resistant to degradation from light and ozone exposure, was included instead of Cy5.

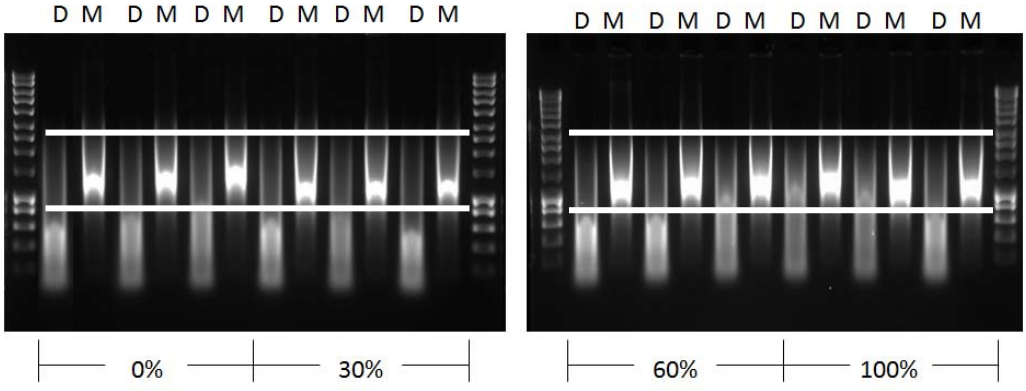


Figure 4.4 Different DNA methylation fractions (0, 30, 60, 100%) were McrBCdigested (D) and mock-digested (M) and resolved on a 1% agarose gel. HyperLadder I was run adjacent to each treated/untreated pair to guide accurate gel slice excision (section cut is highlighted with a white line). Gel was stained with ethidium bromide, run at 40V for 2 hours. 2.5 µg of DNA sample was loaded per lane in a final volume of 20 µl containing loading buffer.

4.2 RESULTS AND DISCUSSION

4.2.1 First Microarray Protocol Validation

In the present investigation we sought to develop a new microarray strategy to query the methylation status of ~12000 CG-rich fragments in Caco-2 cells to determine variations in methylation status after treatment with the bioactive food compounds iberin and/or Se-methylselenocysteine. Initial statistical analysis revealed ~200 genes that were hyper- or hypomethylated in each of the different treatment groups (Figure 4.5).

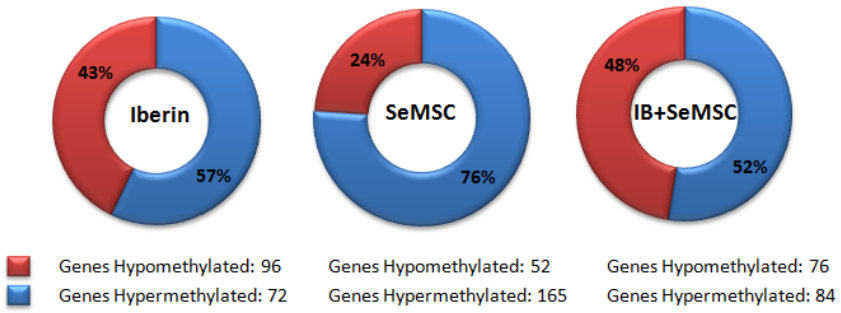


Figure 4.5 Chart displaying numbers of genes with statistically significant epigenetic changes per treatment group.

For the validation process 10 loci from the selenium group that displayed hyper- or hypomethylation changes compared with the control group (FDR *P*-value <0.05) were preliminarily screened to validate if their methylation status corresponded to the same methylation pattern seen in the CpG island microarray. For all the genes evaluated the methylation change was assessed by the COBRA assay as described in section 4.1.2.2.6.1. For instance, Figure 4.6 was obtained after digesting the *IL4R* gene with the restriction enzyme *Hinf* I (Table 4.2) and running the product in 8% polyacrylamide gel before the density of the bands was quantified (Table 4.6). The differences in the percentage of methylation between the control and selenium group were calculated by using the equation shown below Table 4.6.

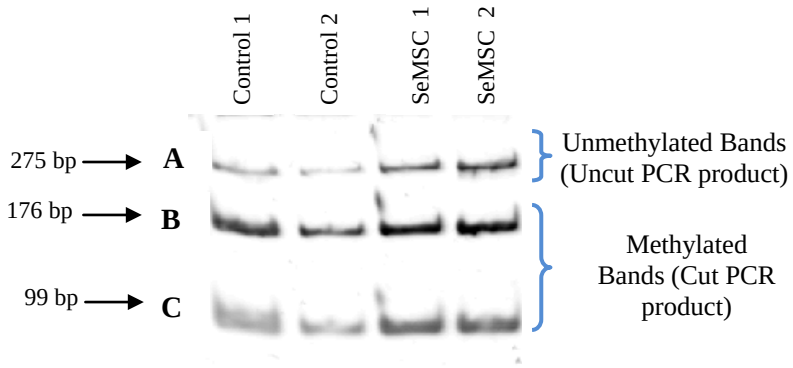


Figure 4.6 COBRA assay showing bands obtained after enzyme digestion. Products from the *IL4R* primary PCR were precipitated prior to an overnight digestion with the *Hinf* I enzyme. The digested DNA was separated in an 8% polyacrylamide gel and stained with SYBR green I before detecting its fluorescence as described in the methods section. The proportion of methylated versus unmethylated DNA was determined from the relative intensities of cut and uncut PCR products.

Table 4.6 Results from IL4R Band Quantification

	Control 1	**Control 2**	**Selenium 1**	**Selenium 2**
Band **A**	558,333.31	98,773.67	712,383.50	1,349,995.33
Band **B**	3,374,606.50	1,380,633.31	3,546,819.56	4,576,974.00
Band **C**	3,641,883.50	1,490,043.00	3,522,149.48	4,394,192.13
% Methylation	92.63	96.67	90.84	86.92
Average	**94.65%**		**88.88%**	

$$\textbf{\% Methylation} = \frac{\text{Band B} + \text{Band C}}{\text{Band A} + \text{Band B} + \text{Band C}} \times 100$$

Methylation Change: 88.88 – 94.65 = -5.77%

The results shown above indicate a reduction in the level of methylation from 95% in the control sample to 89% in the selenium treated group. The same steps were carried out for the remaining 9 genes shown in Table 4.2. However, as shown in Tables 4.7 and 4.8, the results obtained for the COBRA assay differ substantially from the results obtained by the microarray, for instance

PIK4CB and *DVL3* from the hypermethylated list were not methylated as assessed by the COBRA assay. Although some genes used for validation seem to follow the same pattern as the microarray result (*COL1A1, TIRAP, EPHA7, MYST1),* the methylation change obtained with COBRA is markedly different (Table 4.7 and 4.8). For instance, the methylation level of *EPHA7* changed from 10% in control samples to 95% after SeMSC treatment (methylation change= +85% according to the microarray analysis), but after validating this CpG island using COBRA the variation in methylation detected corresponded only to 4% (from 12 to 16%). Furthermore, according to the microarray results observed in the hypomethylated list the *SIRT2* gene was found to decrease in level of methylation from 99 to 88% (methylation change= -11). However, as shown in Table 4.8 this gene showed no methylation by COBRA analysis.

Table 4.7 Candidates Genes from the Hypermethylated Group

Gene Symbol	***Methylation Change***	
	Microarray (%)	**COBRA (%)**
COL1A1	+33	1.01±2.4
TIRAP	+43	5.66±2.5
EPHA7	+85	4.07±4.9
PIK4CB	+58	No Methylation
DVL3	+17	No Methylation
IL4R	+63	-5.77±3.1

Table 4.8 Candidates Genes from the Hypomethylated Group

Gene Symbol	***Methylation Change***	
	Microarray (%)	**COBRA (%)**
SIRT2	-11	No methylation
MYST1	-17	-2.38±5.17
PDCD10	-33	No methylation
DUSP10	-28	No methylation

At first, it was hypothesised that this lack of correlation seen might be due to the COBRA assay sensitivity. Despite the fact that COBRA is a quantitative method to detect methylated alleles, it is not highly sensitive and has been calculated to have a limit of detection of ~1 methylated allele in 200 unmethylated alleles (Belshaw et al. 2004). In addition, this assay has been found to offer a narrow dynamic range leading to underestimation of methylation changes based on strong signals, and secondly the fluorescence background of gel images is often variable (from gel to gel or from lane to lane within the same gel), affecting the calculations and thus making it difficult to reliably compare DNA methylation levels across a sample set (Brena et al. 2006).

In an attempt to identify whether the COBRA assay was hindering the detection of DNA methylation changes due to the previous methodological problems mentioned, we utilised a more robust and sensitive validation method termed Methylation-Sensitive Restriction Enzyme digestion (MSRE) described in section 4.1.2.2.6.2. A difference from the strategy used during the COBRA validation assay, where only genes from the SeMSC group were selected, validation of genome-wide DNA methylation changes using MSRE was based on genes from the different treatment groups (Table 4.9). Additionally, for the majority of genes selected for validation, their methylation change was greater than 50%, with the exception of *ATOH8* and *GALNT7*. From these two genes the former was selected because its CpG island was significantly hypermethylated throughout the different treatments and the latter was chosen to further verify if our methodology was sensitive enough to detect subtle DNA methylation changes (10-20%).

Comparable to the criteria used for COBRA validation, every CpG island considered for MSRE validation had a FDR $P<0.05$ with the exception of *DKFZP564O0823* in which the SeMSC and IB+SeMSC group had a *P* value >0.05 ($P=0.07$), but the iberin group had a *P* value <0.05. In this respect, the first two groups were considered for evaluation as it was the only gene that

showed the same pattern of hypermethylation within the 3 treatment groups. However, these results were interpreted with caution considering their marginal significance levels. In addition, genes identified as hypermethylated (+) or hypomethylated (-) in a single treatment (iberin or SeMSC) and its combination (IB+SeMSC) were selected (*CTNND2, CBX5, FUS*).

Table 4.9 Candidates Genes Selected for Validation and Methylation Changes Obtained Using Microarray and MSRE *

Gene	Iberin		SeMSC		IB+Se	
	Microarray (%)	**MSRE (%)**	**Microarray (%)**	**MSRE (%)**	**Microarray (%)**	**MSRE (%)**
CTNND2	+81	- 5.36± 1.02	-	-	+80	- 3.44± 0.85
CBX5	- 51	No Meth	-	-	- 93	No Meth
FUS	-	-	+54	No Meth	+64	No Meth
ATOH8	+15	No Meth	+16	No Meth	+37	No Meth
DKFZP564O0823	+55	No Meth	+71	No Meth	+87	No Meth
GALNT7	-	-	+21	- 6± 9.35	-	-
CHD2	- 55	- 24±11.61	-	-	-	-
VASP	-	-	-	-	-70	No Meth
PIK4CB	-	-	-	-	+60	- 6.6± 4.27

*Genes showing next to their values a + or – symbol indicates that their CpG island were identified as hypermethylated or hypomethylated respectively when compared with controls. Boxes showing no results within specific treatment groups indicate either a FDR P <0.05 or that the treatment was not selected for validation. Those boxes depicting "No Meth" indicate the CpG island was detected as unmethylated by MSRE.

The results presented in Table 4.9 clearly indicate that the results obtained from the MSRE validation assay did not follow the same pattern observed in the CpG island microarray. This is despite the fact that some of the genes selected were seen to be differentially methylated in two (*CTNND2, CBX5, FUS)* or three (*ATOH8, DKFZP564O0823)* treatments in an attempt to increase the likelihood that the outcome seen at the microarray level for that particular gene was a true effect. In addition, the results provided by the MSRE showed that most of the loci evaluated were unmethylated even though the majority of CpG islands selected for validation had a methylation change according to the microarray analysis of >50%.

4.2.2 Second Microarray

In order to try to identify root causes responsible for the lack of correlation observed between the microarray and the validation process, a second CpG island microarray set-up containing DNA from normal, healthy colon tissue with known methylation percentages was designed to evaluate if the microarray platform employed in this investigation can reliably and accurately detect genome-wide DNA methylation changes. Although the experimental design used for the second microarray differed from the first microarray set-up as a result of adapting the amplicon preparation from (Ordway et al. 2006), the underlying strategy of both microarray platforms, which is based on the enrichment of the unmethylated fraction through the use of the methylation-dependant endonuclease McrBC, remained unaltered.

After analysing the array data and fitting models with methylated and unmethylated components across the different groups to derive a methylation score to be compared with the known percentage of methylation, it was found that the group with 0% of methylated DNA fragments could be interpreted by the modelling as anywhere between 0 and 100% methylation, as the curve of the frequencies for the methylated and unmethylated fraction was almost a normal distribution.. Regarding the rest of the groups containing 30, 60 and 100% of methylated alleles, the estimated methylation score identified with this model corresponded to 60, 67 and 75% respectively (Figure 4.7). These results indicate that small changes in methylation (0-30%) are difficult to detect with this technology. In addition, the fact that the 0% group could be identified as either fully methylated or unmethylated will certainly introduce severe bias to data analysis when an average methylation score for individual CpG islands within this group is derived. Concerning the middle-to-high methylation group, it could be inferred that this methodology can detect with more sensitivity high levels of methylation (60-100%) than low levels (0-30%). However, it was estimated that this technology can detect relatively large changes of methylation levels only when a CpG island goes from low

methylation (<30%) to high methylation (>60%) levels or vice versa,. Smaller changes could only be detected if the level of methylation of a hypermethylated CpG island (>60%) decreased or increased.

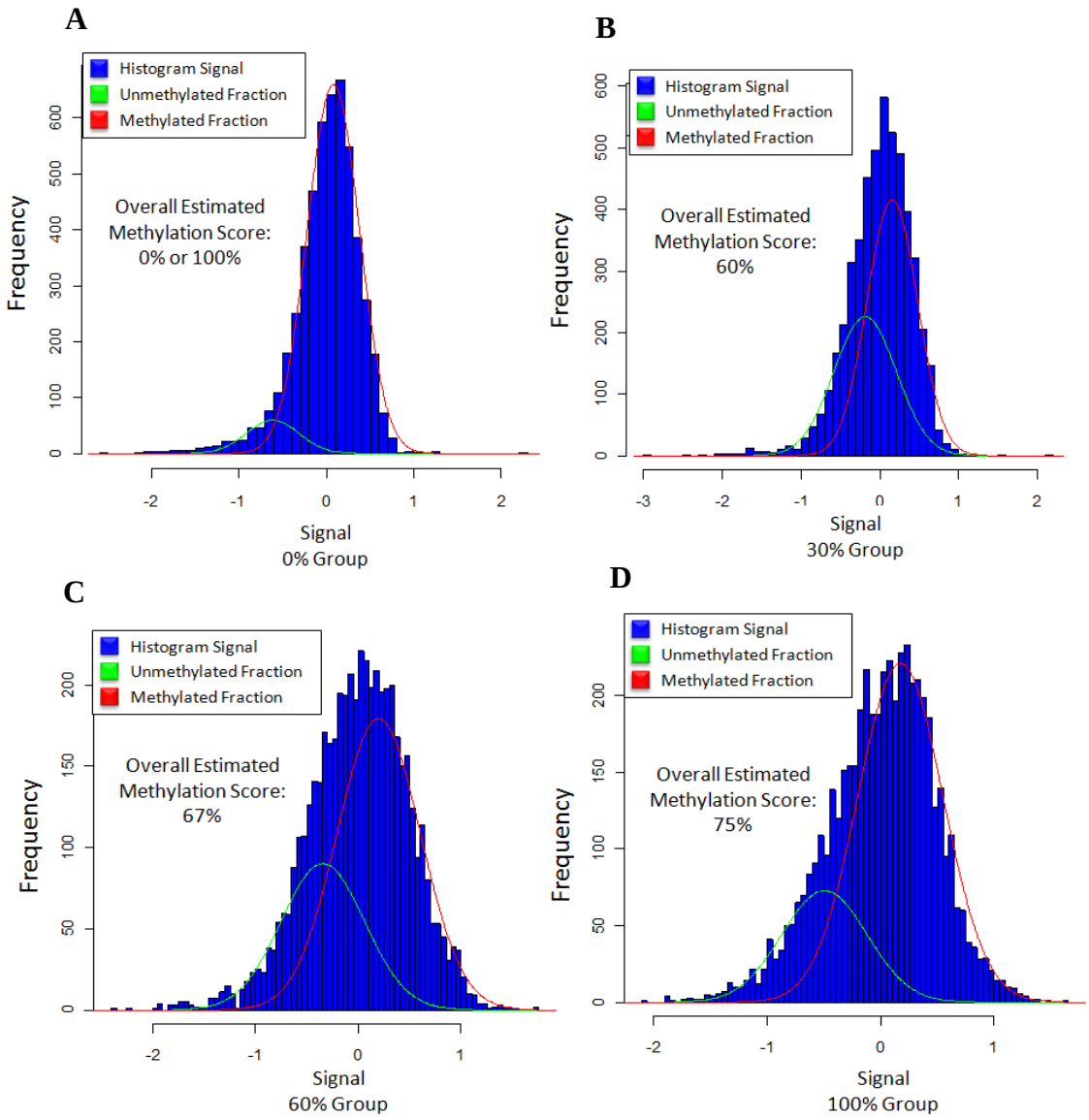


Figure 4.7 Histogram of signals with model overlay from groups with known percentage of methylation: A) 0% B) 30% C) 60% and D) 100%. The blue bars indicate the log-ratio distribution of the methylated (red line) and unmethylated (green line) fractions. The overall methylation score for each fraction is depicted within each graph.

After selecting some CpG islands from the microarray data for further analysis it was observed that although some genes showed a gradual increase in their

methylation status within every artificially generated methylated fraction (Figure 4.8A), other genes showed an increment in their content of 5-methylcytosine only partially as shown in Figure 4.8B, where a continuous enrichment of the methylated fraction was not seen to occur from 60 to 100%. Furthermore, some CpG islands were resistant to augmentation of their methylation status (Figure 4.8C).

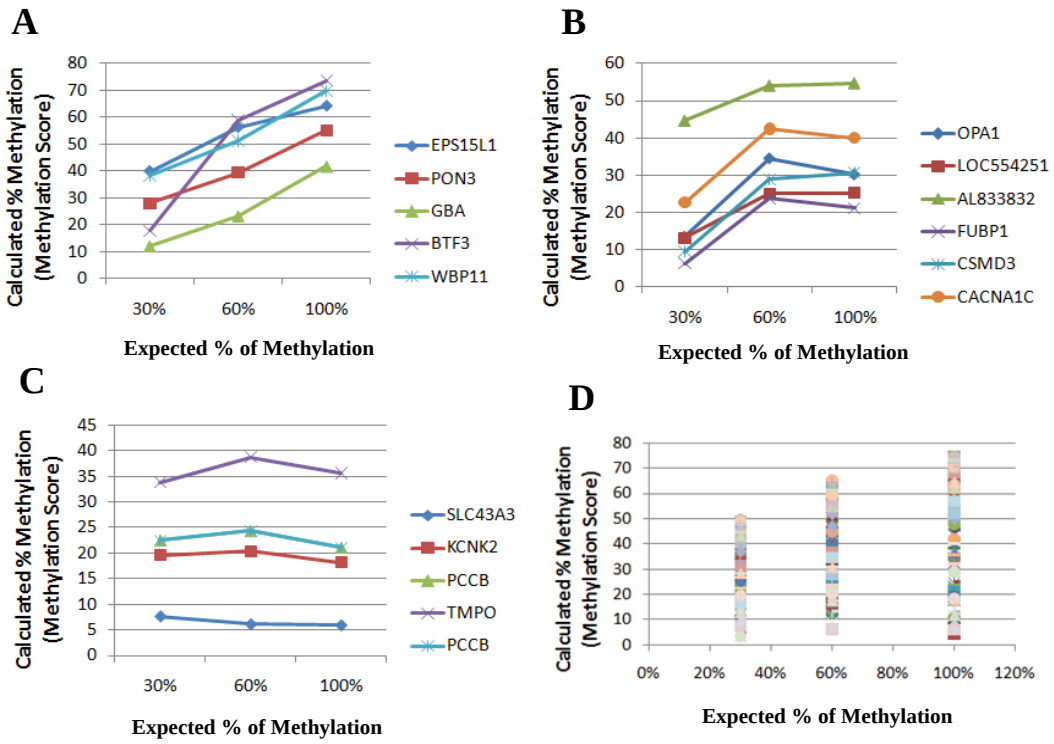


Figure 4.8 Graphs A-C illustrate measured percentage of methylation (methylation scores) for various CpG islands and their expected percentage of methylation based on *in vitro* methylation of human colon DNA. Graph D represents a scatter plot of 150 random CpG islands depicting the inability of the methylation score model and/or microarray platform to predict expected percentages of methylation adequately. The 0% fraction was excluded from the graphs for the reasons mentioned above.

Even though there is a theoretical possibility that the enzyme reaction with M.SssI methylase did not go to completion, the artificially methylated DNA was assessed before we created the different methylated fractions by checking the methylation status of *FUS* by MSRE (one of the genes validated in Section 4.1.2.2.6.2) obtaining 93% methylation. Nevertheless, the artificial methylation

process is not perfect and there are likely to be gene-specific differences in the efficiency of methylation. Finally, some of those genes shown in Figure 4.8B and C might belong to a small proportion of genes that will be normally methylated in noncancerous colon tissue, which are unlikely to be affected by *in vitro* methylation.

Previous studies using microarray-based DNA methylation profiling technologies have tried to ascertain levels of methylation by identifying only hypermethylated or hypomethylated extreme groups with high methylation changes when compared with controls, overlooking potentially relevant subtle DNA methylation changes. However, this research has attempted to derive a quantitative system using the global methylation level of all sequences on the array (i.e area under the curve in Figure 4.7), relative to the total area under the complete log-ratio density curve to estimate individual methylation levels referred to as a methylation score.

Overall, this assay has been shown to be neither accurate nor reproducible across the entire range of possible methylation levels. Figure 4.8D shows the calculated methylation score for 150 random CpG islands plotted against their assumed methylation state and indicates the high variability, lack of natural clustering structure and low levels of precision obtained for the microarray derived methylation score. This analysis indicates that either the algorithms applied do not produce reliable results and/or the microarray platform used does not accurately reflect the methylation status of the sample studied when these algorithms are applied. This analysis suggests that the absence of correlation between the microarray results and the validation data encountered during the first microarray set-up was likely to be due to the inconsistencies with the statistical model and the inherent limitations of the McrBC microarray approach used rather than internal experimental conditions and/or methods selected for validation.

Methylation changes induced by bioactive food compounds have the potential to alter the methylome and shift the abnormal methylation pattern seen during carcinogenesis to that belonging to non-cancerous cells. However, a more comprehensive analysis of the results is required to detect global changes in the cancer epigenome after addition of food compounds to identify, integrate and interpret pathways disrupted in a meaningful manner.

Taken together, there is a need to increase the sensitivity and specificity of the current microarray detection platform used in this investigation. Therefore, further research should be directed towards evaluating suitable statistical models or normalization algorithms in order to identify CpG islands with differential fluorescence ratios that would accurately allow for a more refined recognition of various methylation profiles. A number of microarray-based technologies are already available. However all of these methods have some limitations, which render them unsuitable for some experimental setups (Estecio and Issa 2009).

Additionally, many technological parameters such as the influence of DNA sequence variation, amplification conditions and effective coverage and resolution of enzyme-based array hybridization platforms, which depend on the distribution of potential cleavage sites and the composition of the hybridization array, have recently been investigated to shed light on the advantages and limitations of array-based DNA methylation analyses. These studies have reported that that although the McrBC assay performed the best among other microarray-based platforms such as MeDIP (Weber et al. 2005) and HELP (Khulan et al. 2006), its sensitivity reached only 60%, which makes it vulnerable to the generation of false positive and false negative results (Irizarry et al. 2008; Irizarry et al. 2009). Furthermore, the McrBC method cannot differentiate between unmethylated and polymorphic cytosines. In humans, ~2.16 million SNPs are detected in CpG dinucleotides, and such CpG SNPs are 6.7-fold more abundant than expected (Tomso and Bell 2003). Depending on the restriction enzyme combination used, previous CpG island array-based

studies have demonstrated that 10–30% of all outliers initially detected as methylation differences contained SNPs (Schumacher et al. 2006).

All the previously mentioned drawbacks have resulted in the optimization of tiling array designs incorporating novel genetics smoothing algorithms to measure methylation from raw microarray data resulting in improved performance for McrBC digestion-based techniques. This optimized workflow is referred to as comprehensive high-throughput arrays for relative methylation (CHARM) (Irizarry et al. 2008).

Current emerging technologies in genome-wide methylation profiling as well as the development of computational tools and resources for DNA methylation analysis is accelerating rapidly and promises to revolutionize every field in which, as in genomic research, DNA methylation information is of use (Laird 2010).

5

Discussion & Future Perspectives

5.0 GENERAL DISCUSSION

The present investigation has demonstrated the relevance of combining two bioactive food constituents namely isothiocyanates and selenium in an *in vitro* model of colon cancer using Caco-2 cells to synergistically enhance the expression of specific selenoproteins known to play a major role in controlling redox-regulated processes in colon cells. In addition, this study has investigated the impact of these dietary factors to modulate genome-wide (Line-1) or gene-specific aberrant DNA methylation pattern present in colon cancer cells, observing a lack of effects when administered either individually or in combination in Caco-2 and HCT116 colon cancer cell lines. However, a long-term exposure to these chemicals in diet might potentially lead to an effect on DNA methylation patterns that will ultimately impact upon gene expression and cancer susceptibility. Strategies that use a combinatorial approach of bioactive food components offer an exciting opportunity to strengthen efforts into preventive programs rather than focusing primarily on treatment of end-stage disease. Consequently, results from this research are potentially significant in terms of providing an attractive chemopreventive strategy for future studies applying isothiocyanates and selenium to inhibit or reverse carcinogenesis at its earliest stages.

The association between fruit and vegetables and the risk of colon cancer is complex and has been the focus of a large number of case control and cohort studies with inconsistent and disappointing results to date as discussed in Chapter 1. An interesting hypothesis that may explain the weak or absent associations found in these studies could be that only specific types of fruit and vegetables or their related bioactive constituents/nutrients confer protection against colon cancer risk, suggesting that a protective effect of certain fruit/vegetable subgroups or anticarcinogenic food constituents could be diluted when all of these food groups are considered as a whole.

Until recently most of the research carried out on interaction of diet and genetics has focused mainly on a single dietary factor. However, given the nature of tumour biology and multiple mechanisms involved in colon carcinogenesis, it has been widely recognised that there may be advantages in combining a variety of food constituents to benefit from their complementary mechanisms of action to prevent the accumulation of alterations during neoplastic transformation that lead to uncontrolled cell growth and loss of genomic stability.

Normally, the balance between cell proliferation and apoptosis in colonic mucosa is tightly controlled in order to preserve a constant cell number (Anti et al. 2001). The interruption of this balance results in an escape from the normal homeostasis of colonocytes, which favours the survival of the mutated and undifferentiated cells. Chemoprevention is an attractive concept in colon cancer prevention and its success lies in the understanding of the molecular basis of carcinogenesis. In this respect, food compounds can exert their chemopreventive actions at various stages of colon cancer development by preventing the genotoxic damage of cellular DNA upon exposure to endogenous or exogenous carcinogens (initiation phase), inhibition of clonal expansion of initiated cells by induction of apoptosis and modulation of signal transduction (promotion phase) and blockade of tumour with invasive and metastatic potential (progression phase).

In the light of evidence from previous studies, the present study addressed the anticarcinogenic bioactivities of the glucosinolate hydrolysis products sulforaphane or iberin either individually or in combination with an inorganic (selenite) or organic (SeMSC) form of the mineral selenium to elucidate plausible mechanisms of action to prevent colon cancer at key stages in the cancer process. For example, results presented in this thesis indicate that after combining the ITCs and selenium, sulforaphane and selenite were the most effective chemical forms that elicit a greater time- and dose-dependent increase in the expression of the selenoproteins *TrxR1* and *GI-GPx* than either

compound alone. The importance of these findings relate to the fact that in the pre-initiation/early stages of colon carcinogenesis the disruption of cellular defense mechanisms (consisting of a battery of detoxifying or antioxidant enzymes) would make cells/tissues more susceptible to DNA damage by both unwanted by-products of normal cellular metabolism, or other environmental sources of ROS, which in rapidly dividing cells, e.g., the epithelial cell of the colon, can escape repair mechanisms resulting in somatic mutations (Stone et al. 2004). Therefore, an increase in the cellular antioxidant defense mechanisms may prevent the deleterious effects of free radicals that would otherwise affect important biomolecules and render colon cells to accumulation of genetic alterations that lead to cancer progression.

Isothiocyanates are promising chemopreventive agents that have been shown to activate the redox-sensitive Keap1-Nrf2 signaling pathway involved in the transcriptional activation of genes encoding phase II enzymes and selenoproteins such as TrxR1 and GI-GPx. This activation normally takes place through the antioxidant response element (ARE) present in their promoter region. To investigate whether the mechanisms associated with the synergistic induction of these selenoprotein after simultaneous addition of ITC+Se was attributed to Nrf2 activation, nuclear protein extracts obtained after culturing Caco-2 cells with these food compounds indicated that this synergistic up-regulation was not Nrf-2 dependent, indicating that selenium can delay the degradation of ITCs-induced TrxR1 and GI-GPx mRNA, favouring therefore more protein synthesis as indicated in section 2.2.3.1.

Furthermore, after conducting time- and dose-dependent experiments, contrasting mechanisms of regulation were observed for each individual selenoprotein at the mRNA level. Whilst TrxR1 was shown to respond in a synergistic manner after addition of ITCs+Se at both the transcriptional and translational level, the GI-GPx transcript remained unaltered after co-addition of selenium. In contrast, a clear up-regulation of GI-GPx after supplementing cells with ITC+Se was observed at the protein level. This unchanged or stable

expression of GI-GPx at the transcriptional level ranks this selenoenzyme high in the hierarchy of selenoproteins in colon cells (Banning et al. 2005), indicating that under limited selenium supply conditions the mRNA of GI-GPx can even increase and become preferentially translated when selenium supply is restored again (Brigelius-Flohe 2006). The contrasting results of GI-GPx and TrxR1 mRNA regulation obtained in this study indicate that the former may have more vital functions in colon cancer cells than the latter as the relative position of selenoproteins within its hierarchy is believed to reflect their relative biological importance within the studied system. However, although the ranking of TrxR1 may be lower in colon cancer cells, others have shown that over-expression of TrxR1 in human embryonic kidney cells (HEK-293) led to a decrease in GPx1, indicating that enhanced TrxR1 transcripts withdraw selenium from the biosynthesis of less important selenoproteins (Nalvarte et al. 2004). This suggests that selenoproteins are regulated differently across different cells and/or tissues. Interestingly, the factor that distinguishes between the different selenoprotein mRNAs to elicit varying expression levels of the corresponding proteins is the SECIS-binding protein 2 (SBP2), a factor required for incorporation of selenium into selenoproteins (Berry 2005).

The contribution of oxidative stress towards the aetiology of colon cancer has been recognised widely. It is well known that incidence of colon cancer is at least 30-fold higher than that of the small intestine (Chadwick et al. 1992). In contrast to the small intestine, the colon is exposed to high levels of superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2) and other powerful mutagenic reactive nitrogen species as a result of the presence of fecal bacteria and bile pigments (Babbs 1990; Bernstein et al. 2005; Valko et al. 2006). These oxidants, under normal conditions, are scavenged efficiently, but when their production outstrips antioxidant protection mechanisms (e.g., inflammatory bowel diseases or ageing), the accumulation of somatic mutations can give rise to colon cancer. As a result, the ratio of glutathione and oxidised glutathione (GSH/GSSG), which is a good

measure of oxidative stress (Hwang et al. 1992), has been found to be reduced significantly in the blood of patients with colon cancer (Pastore et al. 2003).

Important findings in this thesis show that after challenging Caco-2 cells with the endogenous oxidant H_2O_2, simultaneous addition of sulforaphane (6 µM) plus selenite (50 nM) evoked greater cell protection from hydrogen peroxide-induced cell injury than when either compound was added in isolation. However, sulforaphane is one of the most powerful dietary inducers of several genes containing an ARE in their promoter region such as phase II enzymes, redox-active proteins, GSH-related enzymes and several other novel enzymes recently identified (Malhotra et al. 2010). Therefore, in order to investigate whether the protection exerted by sulforaphane and selenite after H_2O_2 treatment was driven by TrxR1 and GI-GPx, or if other Nrf2-dependent/antioxidant genes may have influenced the results, Caco-2 cells were transfected with siRNA to generate a single and double knock-out approach by using *TrxR1* and *GI-GPx* siRNA before addition of food constituents and H_2O_2. The results demonstrated that both selenoproteins were essential to preserve cell integrity after a significant reduction in cell viability in the double knock-out model, similar to that observed in control cells without treatment, was observed.

Increasing understanding of the effect of isothiocyanates and selenium on TrxR1 and GI-GPx expression has many implications. For example, previous studies have supported the notion that expression of selenoproteins can be mutually exclusive as shown by the contrasting expression patterns of GPx1 and TrxR1 in both human and mouse cells (Gladyshev et al. 1998), as well as of GI-GPx and GPx3 or selenoprotein P in gastrointestinal cancers (Mörk et al. 2000; Mörk et al. 2003). However, our results suggest that once these selenoenzymes have been stimulated synergistically by the studied dietary factors they will act in concert under oxidative stress conditions (regardless of their selenoprotein hierarchy) in order to re-establish the delicate intracellular redox balance. This adds another level of regulation previously unknown.

Thus, ITC+Se-mediated synergistic selenoprotein protection might be beneficial to avoid cancer initiation in normal colonocytes and might be achieved through the incorporation in the diet of specific food sources rich in both of these bioactive food compounds. In this respect studies have shown that broccoli grown on selenium-fertilized soil inhibited the development of chemically induced preneoplastic lesions in rat colon (Finley et al. 2000), of spontaneous development of intestinal tumours in mice (Davis et al. 2002) and of mammary tumours in rats (Finley et al. 2001).

The concentrations of selenium used in this study (25 to 200 nM) are thought to maximise the activity of the seleno-dependent enzymes assessed in this study using Caco-2 cells grown in low selenium cell culture media (16 nM). Although these selenium concentrations used are low when compared to the average selenium concentrations found in humans (between 1 and 5 µM) (Gallegos et al. 1997) following a mean selenium intake of 60-75 µg per day (reference nutrient intake for selenium, UK) and above, the total available selenium in cell culture is determined not only by the selenium concentrations but also the volume of medium or the ratios of medium to number of cells. This *in vitro* study aimed primarily to investigate critical doses and forms of selenium to determine their biochemical, molecular and cellular responses in the regulation of antioxidant enzymes (TrxR1 and GI-GPx). In addition, the aim was to establish mechanistic associations among different dietary constituents that can provide useful insights to formulate further hypotheses that can be later tested *in vivo* or through the design of appropriate intervention studies to decipher their potential anticancer activities. For instance, the results presented in this thesis shed light into the potential mechanisms responsible for the decreased tumour incidence observed in mice by Finley and colleagues after administering high selenium broccoli.

However, regarding the incorporation of dietary constituents to prevent cancer mentioned above, the biochemistry underlying associations between diet and cancer is exceptionally complex, and the response to specific nutrients and

specific dietary components will likely differ depending on a range of factors including an individual's genotype. For instance, individuals with common deletion polymorphisms in Glutathione S-transferases M1 and T1 (*GSTM1* and *GSTT1*) might benefit more in terms of cancer protection from a diet rich in isothiocyanates than those with non-null genotype as the former may accumulate higher ITC levels in cells/tissues due to their lower rates of ITC excretion (Lin et al. 1998; London et al. 2000).

Data presented in Chapter 3 refer to the influence of isothiocyanates and selenium compounds on DNA methylation in a gene-specific and global manner. Key studies from different experimental models indicate that epigenetics abnormalities may take place during the early stages of neoplastic progression (Jones and Baylin 2007). This particular characteristic and the distinctive methylation profile pattern among tumours (tumour-type specificity) (Esteller et al. 2001), make it an excellent cancer biomarker and the target for both diet-derived chemopreventive agents and targeted chemotherapy. Unfortunately, the applicability of the commonly used chemotherapy treatment 5-Aza-2'-deoxycytidine (decitabine) for cancer patients is hampered by its highly toxicity, side effects and instability in physiological solutions (Esteller 2005). As a result, there has been considerable interest in the development of effective and non-toxic inhibitors of DNMTs not only for therapy but also for chemoprevention. In this respect, the chemical components of edible fruits and vegetables are promising chemotherapeutic agents that have been widely used in an attempt to reverse abnormal DNA hypermethylation patterns in cancer and restore the expression of silenced genes.

Although the role of selenium as an integral part of cellular antioxidant enzymes and as an essential factor for regulating a multitude of cell signalling pathways in cancer has been studied extensively (Lu et al. 2009), there is still significant debate as to what mechanisms account for the anticancer activity of selenium in humans. A plausible mechanism of action might involve alteration of epigenetic signals in gastrointestinal neoplasia through inhibition of DNMT

activity. Likewise, isothiocyanates remain relatively unexplored within the field of DNA methylation as the focus has been mainly diverted towards their impact upon chromatin remodelling factors.

In this study we have evaluated the ability of different forms of selenium (including SeMSC as it is currently thought to offer the most promise as potential anticarcinogenic among selenium compounds) and isothiocyanates to influence DNA methylation processes. These data show that following exposure of Caco-2 and HCT116 cells to different concentrations of sulforaphane and iberin (ranging from 6 to 8 μM) or SeMSC and selenite (ranging from 0.2 to 5 μM) either alone or in combination to investigate their impact on both genome-wide (LINE-1) and gene-specific DNA methylation, no significant changes were observed. Only a transient change in DNMT mRNA expression, which occurred mostly in the treatment groups containing isothiocyanates, was observed. However, these changes that took place only for specific DNMT transcripts and did not modify the aberrant methylation status present in the cells, e.g. coexistence of global hypomethylation and local gene-specific hypermethylation. These results suggest that dietary compounds would have to target preferentially the activity of all the principal DNA methyltransferase enzymes in order to affect the methylation of cytosines at CpG dinucleotides. The observation may also indicate that in most cells different DNMTs cooperate with one another at all levels to propagate DNA methylation changes, which is in line with the current model suggested for the establishment and inheritance of DNA methylation patterns (Jones and Liang 2009).

In agreement with our observations, a study investigating the impact of 12 dietary phytochemicals on CpG hypermethylation in the MCF7 breast cancer cell line showed no reduction in the DNA methylation status of *RASSF1A*, *GSTP1* and *HIN-1*. Further, all the phytochemicals inhibited the global DNA methyltransferase activity (*de novo* and maintenance), but neither of them affected the DNMT1 transcript nor its protein level, with the exception of

rosmarinic acid (Paluszczak et al. 2010). In contrast, another study showed that genistein (one of the major isoflavones present in soy) caused CpG demethylation and active histone modification of the tumour suppressor gene *BTG3* in different renal cancer cell lines after inhibiting protein levels and activity of both DNMT1 and DNMT3. In addition, a decreased methyl-CpG-binding domain protein 2 (MBD2) and increased histone acetyltransferase (HAT) activity was observed (Majid et al. 2009). Together these data suggest that dietary cancer chemopreventive agents must target key components of the epigenetic machinery to effectively re-establish the expression of silenced genes.

Since up-regulation of GI-GPx and TrxR1 by co-treatment of ITCs and selenium was observed in the early stages of this investigation, it was initially hypothesised that changes, if any at all, in the methylation status of the target genes studied after adding ITCs and/or Se to the cells could be related to a decreased oxidative stress, and considering that previous studies have linked oxidative stress with an increased expression of DNMT, it was a feasible proposal. For instance, Campo et al. suggested that oxidative stress regulates DNA methylation through DNA methyltransferase expression modifications in murine nontumourigenic melanocyte lineage (Campos et al. 2007). Additionally, renal cancers induced by oxidative damage in a rat model were found to have a relatively high rate of methylation associated inactivation of the gene *p16* (33.3%) (Tanaka et al. 1999). Likewise, oxidant-induced transformation of fibroblasts was observed to be associated with an unusual pattern of DNA methylation in an intronic region of the *c-abl* gene (Cerda and Weitzman 1997). However, no changes at the DNA methylation level of the genes studied were observed in response to these food compounds to add support to this hypothesis.

Chapter 4 shows an attempt to design a CpG island microarray together with tailored bioinformatics analysis to unveil DNA methylation changes at specific loci using a methylation-specific restriction enzyme methodology. In view of

the fact that current assessment of genome-wide CpG islands relies mostly on the identification of significantly hypermethylated or unmethylated DNA with elevated methylation differences relative to control and considering that nutritional research often generates subtle changes in DNA methylation that will not be detected accurately by current statistical approaches we sought to develop a new methodology to maximise the number of assayed CpG islands by generating a methylation score for each locus. However, the sensitivity and specificity of the algorithms and/or technique used to detect differentially methylated DNA were found to be low after validation.

Since only selected sites targeted by the restriction enzyme of use can be studied in the technology applied in this study, it has been recognized that the implementation of restriction-enzyme-based methods can be biased to genome compartments (for example CG-rich versus CG-poor areas). However, recently developed methodologies applied to whole human genome DNA methylation analysis as well as novel statistical procedures and array design algorithms have circumvented these limitations and promise to revolutionise the field of methylation. For instance, early analytical difficulties associated with an inability to estimate absolute methylation levels in immunoprecipitation-based methods for DNA methylome analysis, such as MeDIP, (Weber et al. 2005) have been currently bypassed by transforming normalised MeDIP-chip $\log_2$-ratios into a quantitative measure of DNA methylation across a wide range of CpG densities in an algorithm tool termed Bayesian tool for methylation analysis (Batman) (Down et al. 2008). This analytical tool represents a suitable statistical platform that can be employed to measure subtle statistical changes in future nutritional studies and is more sensitive than the previous analytical tool mentioned in Chapter 4 termed CHARM (Irizarry et al. 2008), which requires the use of a particular array design and does not estimate absolute DNA methylation levels and suffers to some degree in the ability to discriminate highly methylated from highly unmethylated CpG islands (Down et al. 2008).

5.1 FUTURE PERSPECTIVES

The chemopreventive effects of cruciferous vegetables and selenium compounds have been studied widely, demonstrating strong cancer-preventive activity *in vitro* and *in vivo* but inconsistent levels of protection has been reported in intervention human studies. Despite significant progress in our understanding of multistage carcinogenesis, much remains to be known in relation to the mechanisms of action of most chemopreventive agents and their potential effectiveness.

Although growing evidence from the literature supports the hypothesis that antioxidant enzymes protect against colon cancer by means of counteracting ROS levels to maintain physiological homeostasis, the overall picture is far from understood. Data emerging from *in vivo* and *in vitro* studies suggest that TrxR1 and GI-GPx are up-regulated in cancer. These observations provide evidence that TrxR1 is critical for self-sufficiency in growth of malignant cells, in which this selenoprotein acts predominantly as a pro-cancer protein (Yoo et al. 2007). Likewise, GI-GPx mRNA has been found elevated in human colorectal adenomas and carcinomas (Lin et al. 2002), and appears to support the proliferation of established cancer cells. Consequently, simultaneous inhibition of more than one antioxidant system (including TrxR1 and GSH/GSSG) has recently been shown to be a promising target for cancer therapy (Mandal et al. 2010).

In the light of findings from previous studies it is most likely that results from this research may be potentially exploited as a cancer prevention treatment before the tumour process has been initiated. Therefore, results obtained from this study should be replicated employing normal parental cell lines as well as cancer cell lines to compare the level of expression of TrxR1 and GI-GPx after co-addition of ITCs+Se and establish similarities with the data gathered here. Also, identification of ROS using H_2DCFDA (a ROS-sensitive fluorescent dye) to confirm the importance of the synergistic up-regulation of both

selenoenzymes on their ROS scavenging activity will be imperative in addition to the study of the protective effects of ITC+Se against ROS-induced DNA strand breakage by employing the comet assay to understand different aspects of protection exerted by these important bioactive dietary compounds and their associated selenoprotein up-regulation.

Previous studies have used mice with combined disruption of *GPx1* and *GI-GPx*, resulting in the development of mice with inflammatory bowel diseases, a risk factor for colon cancer (Esworthy et al. 2001) to assess the importance of selenoproteins. New mouse model systems could be devised to examine the regulation of *TrxR1* and *GI-GPx* in the colon of mice restricted to diets supplemented with nutritionally relevant amounts of ITCs or Se, either individually or in combination, to assess the protective effect against intestinal cancer susceptibility in response to treatment with the chemical carcinogen 1,2-dimethylhydrazine. This study would be useful to establish important associations between the level of selenium concentrations in the plasma and liver, glutathione peroxidase enzyme activity, selenoprotein expression in colonocytes and number of tumours developed. In addition, since a greater understanding of the role of diet on DNA methylation in cancer will likely be gained from genome-wide studies and considering the absence of studies of this kind in the literature to date, samples from such a study could be assessed by CpG island microarray using a more robust microarray analysis platform such as MeDIP-Batman to decipher the methylome of healthy and diseased mouse colon tissues.

In addition, taking into account that the selenoprotein TrxR1 carries a putative CpG island close to the transcriptional initiation site (Rundlof et al. 2001) that has not been characterised as affected by CpG island methylation to date, and the influence of promoter polymorphism of *Nrf2* on aberrant DNA methylation in gastric epithelium (Arisawa et al. 2008) in addition to the recently recognised role of DNA methylation mechanisms controlling the expression of *Nrf2* in TRAMP prostate tumours (Yu et al. 2010), it is an attractive idea to

identify if these genes are regulated by ITCs and/or Se with regard to epigenetic mechanisms in colon cancer.

Although it has been generally accepted that, among the mechanisms controlling epigenetic changes, DNA methylation prevails over histone modifications (Esteller 2005), it has recently become apparent that both pathways can be dependent on one another (Cedar and Bergman 2009). Moreover, it has been found recently that DNA hypomethylation is necessary but not sufficient for gene reactivation. Rather, local chromatin structure modification is a key determinant of actual gene re-expression (Si et al. 2010). In this respect, it would be valuable to carry out determination of gene expression in control and treated samples, even though the DNA methylation status of specific genes is not decreased by specific bioactive food compounds, to identify changes in transcript levels that might be potentially related to changes in chromatin architecture. In addition, it would be essential to measure both protein and activity of DNMTs to confirm the results obtained at the mRNA level.

To date, relatively few epidemiological association studies have examined the effects of consumption of cruciferous vegetables on risk of developing cancer and precancerous lesions (IARC/WHO 2004; Zhang 2004; Herr and Büchler 2010). Most epidemiological association studies investigating the relationship between diet and cancer have relied on dietary information collected with food frequency questionnaires, which suffer from recall bias that can lead to inaccurate exposure estimation. Considering the recent development of stable biomarkers that can be used as potential surrogate markers for the long term effect of ITCs at the cellular level, and which may more accurately reflect longer term exposure to ITCs (Kumar and Sabbioni 2010; Kumar et al. 2010), new case-control studies can be planned on the effect of dietary ITC intake and colon cancer. Individuals could be categorised for *GSTM1* and *GSTT1* polymorphisms, and stratified according to their level of plasma/serum selenium and/or selenium intake to detect potentially significant associations

between cruciferous vegetables, selenium status/intake and risk for colon cancer. In addition, long-term cohort studies, with large and well-powered sample sizes and controlling for important lifestyle confounding variables are recommended to investigate and confirm previously reported anti-cancer effects of cruciferous vegetables and/or selenium. Investigations into the potential synergistic effects of isothiocyanates and/or selenium in humans, taking advantage of newly developed biomarkers for ITCs (Kumar et al. 2010) and/or the establishment of more sensitive selenium status biomarkers, such as SePP (Fairweather-Tait et al. 2010), together with standardisation in methodology, would assist in the comparison of studies and also contribute critical information to improve our understanding of the chemopreventive mechanisms of ITC and/or Se.

Finally, whilst this study has shown that SeMSC was more effective than selenite in maintaining a sustained selenoprotein up-regulation after 48 h of treatment, others have hypothesised that although selenite and SeMSC induce GPx1 more rapidly than SeMet (Zeng et al. 2008) the latter is retained and non-specifically incorporated into proteins and might be used in the long term for selenoprotein biosynthesis. Therefore, further studies are warranted to elucidate this issue by using different forms and concentrations of selenium to confirm the data presented in this investigation and confirm the author's hypothesis.

Taken together, the experiments carried out in this thesis have provided novel insights into potential mechanisms of cancer chemoprevention by sulforaphane and selenium both individually and synergistically through regulation of key selenoproteins responsible for the removal of damaging reactive molecules, which are implicated in the progression and development of colon cancer. However, future studies should address both the potential cancer prevention activity of antioxidant enzymes such as TrxR1 and GI-GPx in addition to their contrasting role in the promotion of cancer. Results from these studies will undoubtedly help in refining the optimal intakes of selenium and cruciferous vegetables to prevent cancer development in the future. In addition, the precise role of these food constituents in the aberrant methylation that accompanies

tumorigenesis needs to be further addressed in light of the fact that there are regions of the genome that are methylation-prone and methylation-resistant (Feltus et al. 2003). Although the factors that contribute to methylation susceptibility are not completely known, this phenomenon rises the question of whether there are regions of the epigenome that are more susceptible than others to dietary compounds that could explain the lack of effect of ITCs and selenium observed in this study. Therefore, the influence of these bioactive compounds on factors affecting the complete epigenetic setting of the transformed cell, including DNA methylation, chromatin remodelling factors, histone modifications and CpG binding proteins are warranted to decipher their impact on DNA methylation patterns in colon cancer.

REFERENCES

Abbaszadegan MR, Moaven O, Sima H, Ghafarzadegan K, A'Rabi A, Forghani MN, Raziee HR, Mashhadinejad A, Jafarzadeh M, Esmaili-Shandiz E and Dadkhah E (2008). "p16 promoter hypermethylation: a useful serum marker for early detection of gastric cancer." World J Gastroenterol **14**(13): 2055-60.

Acheson AG and Scholefield JH (2008). "Survival from cancers of the colon and rectum in England and Wales up to 2001." Br J Cancer **99**(S1): S33-S34.

Aherne SA and O'Brien NM (1999). "Protection by the Flavonoids Myricetin, Quercetin, and Rutin Against Hydrogen Peroxide-Induced DNA Damage in Caco-2 and Hep G2 Cells." Nutrition and Cancer **34**(2): 160 - 166.

Al-Taie OH, Uceyler N, Eubner U, Jakob F, Mork H, Scheurlen M, Brigelius-Flohe R, Schottker K, Abel J, Thalheimer A, Katzenberger T, Illert B, Melcher R and Kohrle J (2004). "Expression Profiling and Genetic Alterations of the Selenoproteins GI-GPx and SePP in Colorectal Carcinogenesis." Nutrition and Cancer **48**(1): 6-14.

Alberts DS, Einspahr JG, Krouse RS, Prasad A, Ranger-Moore J, Hamilton P, Ismail A, Lance P, Goldschmid S, Hess LM, Yozwiak M, Bartels HG and Bartels PH (2007). "Karyometry of the Colonic Mucosa." Cancer Epidemiol Biomarkers Prev **16**(12): 2704-2716.

Amoako PO, Uden PC and Tyson JF (2009). "Speciation of selenium dietary supplements; formation of S-(methylseleno)cysteine and other selenium compounds." Anal Chim Acta **652**(1-2): 315-23.

Andelova H, Rudolf E and Cervinka M (2007). "In vitro antiproliferative effects of sulforaphane on human colon cancer cell line SW620." Acta Medica (Hradec Kralove) **50**(3): 171-6.

Ang P, Loh M, Liem N, Lim P, Grieu F, Vaithilingam A, Platell C, Yong W, Iacopetta B and Soong R (2010). "Comprehensive profiling of DNA methylation in colorectal cancer reveals subgroups with distinct clinicopathological and molecular features." BMC Cancer **10**(1): 227.

Anti M, Armuzzi A, Morini S, Iascone E, Pignataro G, Coco C, Lorenzetti R, Paolucci M, Covino M, Gasbarrini A, Vecchio F and Gasbarrini G (2001). "Severe imbalance of cell proliferation and apoptosis in the left colon and in the rectosigmoid tract in subjects with a history of large adenomas." Gut **48**(2): 238-46.

Aparicio A, North B, Barske L, Wang X, Bollati V, Weisenberger D, Yoo C, Tannir N, Horne E, Groshen S, Jones P, Yang A and Issa JP (2009). "LINE-1 methylation in plasma DNA as a biomarker of activity of DNA methylation inhibitors in patients with solid tumors." Epigenetics **4**(3): 176-84.

Arikawa AY and Gallaher DD (2008). "Cruciferous Vegetables Reduce Morphological Markers of Colon Cancer Risk in Dimethylhydrazine-Treated Rats." J. Nutr. **138**(3): 526-532.

Arisawa T, Tahara T, Shibata T, Nagasaka M, Nakamura M, Kamiya Y, Fujita H, Yoshioka D, Arima Y, Okubo M, Hirata I and Nakano H (2008). "The influence of promoter polymorphism of nuclear factor-erythroid 2-related factor 2 gene on the aberrant DNA methylation in gastric epithelium." Oncol Rep **19**(1): 211-6.

Arnér ES (2009). "Focus on mammalian thioredoxin reductases--important selenoproteins with versatile functions." Biochim Biophys Acta **1790**(6): 495-526.

Babbs CF (1990). "Free radicals and the etiology of colon cancer." Free Radical Biology and Medicine **8**(2): 191-200.

Bacon JR, Williamson G, Garner RC, Lappin G, Langouet S and Bao Y (2003). "Sulforaphane and quercetin modulate PhIP-DNA adduct formation in human HepG2 cells and hepatocytes." Carcinogenesis **24**(12): 1903-1911.

Banning A, Deubel S, Kluth D, Zhou Z and Brigelius-Flohe R (2005). "The GI-GPx Gene Is a Target for Nrf2." Mol. Cell. Biol. **25**(12): 4914-4923.

Bao Y, Jemth P, Mannervik B and Williamson G (1997). "Reduction of thymine hydroperoxide by phospholipid hydroperoxide glutathione peroxidase and glutathione transferases." FEBS Letters **410**(2-3): 210-212.

Bariol C, Suter C, Cheong K, Ku S-L, Meagher A, Hawkins N and Ward R (2003). "The Relationship between Hypomethylation and CpG Island Methylation in Colorectal Neoplasia." Am J Pathol **162**(4): 1361-1371.

Basten GP, Bao Y and Williamson G (2002). "Sulforaphane and its glutathione conjugate but not sulforaphane nitrile induce UDP-glucuronosyl transferase (UGT1A1) and glutathione transferase (GSTA1) in cultured cells." Carcinogenesis **23**(8): 1399-404.

Baylin SB (2005). "DNA methylation and gene silencing in cancer." Nat Clin Pract Oncol **2 Suppl 1**: S4-11.

Beckett GJ and Arthur JR (2005). "Selenium and endocrine systems." J Endocrinol **184**(3): 455-65.

Bédrine-Ferran H, Le Meur N, Gicquel I, Le Cunff M, Soriano N, Guisle I, Mottier S, Monnier A, Teusan R, Fergelot P, Le Gall J-Y, Léger J and Mosser J (2004). "Transcriptome variations in human CaCo-2 cells: a model for enterocyte differentiation and its link to iron absorption." Genomics **83**(5): 772-789.

Bellinger FP, Raman AV, Reeves MA and Berry MJ (2009). "Regulation and function of selenoproteins in human disease." Biochem J **422**(1): 11-22.

Belshaw NJ, Elliott GO, Foxall RJ, Dainty JR, Pal N, Coupe A, Garg D, Bradburn DM, Mathers JC and Johnson IT (2008). "Profiling CpG island field methylation in both morphologically normal and neoplastic human colonic mucosa." Br J Cancer **99**(1): 136-42.

Belshaw NJ, Elliott GO, Williams EA, Bradburn DM, Mills SJ, Mathers JC and Johnson IT (2004). "Use of DNA from Human Stools to Detect Aberrant CpG Island Methylation of Genes Implicated in Colorectal Cancer." Cancer Epidemiol Biomarkers Prev **13**(9): 1495-1501.

Belshaw NJ, Elliott GO, Williams EA, Mathers JC, Buckley L, Bahari B and Johnson IT (2005). "Methylation of the ESR1 CpG island in the colorectal mucosa is an 'all or nothing' process in healthy human colon, and is accelerated by dietary folate supplementation in the mouse." Biochem. Soc. Trans. **33**(Pt 4): 709-711.

Benz CC and Yau C (2008). "Ageing, oxidative stress and cancer: paradigms in parallax." Nat Rev Cancer **8**(11): 875-9.

Bernstein C, Bernstein H, Payne CM, Dvorak K and Garewal H (2008). "Field defects in progression to gastrointestinal tract cancers." Cancer Lett **260**(1-2): 1-10.

Bernstein H, Bernstein C, Payne CM, Dvorakova K and Garewal H (2005). "Bile acids as carcinogens in human gastrointestinal cancers." Mutation Research/Reviews in Mutation Research **589**(1): 47-65.

Berry MJ (2005). "Insights into the hierarchy of selenium incorporation." Nat Genet **37**(11): 1162-1163.

Bertl E, Bartsch H and Gerhauser C (2006). "Inhibition of angiogenesis and endothelial cell functions are novel sulforaphane-mediated mechanisms in chemoprevention." Mol Cancer Ther **5**(3): 575-85.

Bjelakovic G, Nikolova D, Simonetti RG and Gluud C (2008). "Systematic review: primary and secondary prevention of gastrointestinal cancers with antioxidant supplements." Alimentary Pharmacology & Therapeutics **28**(6): 689-703.

Boffetta P, Couto E, Wichmann J, Ferrari P, Trichopoulos D, Bueno-de-Mesquita HB, van Duijnhoven FJB, Buchner FL, Key T, Boeing H,

Nothlings U, Linseisen J, Gonzalez CA, Overvad K, Nielsen MRS, Tjonneland A, Olsen A, Clavel-Chapelon F, Boutron-Ruault M-C, Morois S, Lagiou P, Naska A, Benetou V, Kaaks R, Rohrmann S, Panico S, Sieri S, Vineis P, Palli D, van Gils CH, Peeters PH, Lund E, Brustad M, Engeset D, Huerta JM, Rodriguez L, Sanchez M-J, Dorronsoro M, Barricarte A, Hallmans G, Johansson I, Manjer J, Sonestedt E, Allen NE, Bingham S, Khaw K-T, Slimani N, Jenab M, Mouw T, Norat T, Riboli E and Trichopoulou A (2010). "Fruit and Vegetable Intake and Overall Cancer Risk in the European Prospective Investigation Into Cancer and Nutrition (EPIC)." J. Natl. Cancer Inst. **102**(8): 529-537.

Branham WS, Melvin CD, Han T, Desai VG, Moland CL, Scully AT and Fuscoe JC (2007). "Elimination of laboratory ozone leads to a dramatic improvement in the reproducibility of microarray gene expression measurements." BMC Biotechnol **7**: 8.

Brena RM, Auer H, Kornacker K, Hackanson B, Raval A, Byrd JC and Plass C (2006). "Accurate quantification of DNA methylation using combined bisulfite restriction analysis coupled with the Agilent 2100 Bioanalyzer platform." Nucleic Acids Res **34**(3): e17.

Brigelius-Flohe R (2006). "Glutathione peroxidases and redox-regulated transcription factors." Biological Chemistry **387**(10/11): 1329-1335.

Brigelius-Flohe R and Banning A (2006). "Part of the Series: From dietary antioxidants to regulators in cellular signaling and gene regulation." Free Radical Research **40**(8): 775 - 787.

Brigelius-Flohé R and Banning A (2009). Dietary factors in the regulation of seleneprotein biosynthesis. In: Dietary Modulation of Cell Signaling Pathways Surh Y.-J. Boca Raton, CRC Press**:** p. 251-271.

Brigelius-Flohé R and Kipp A (2009). "Glutathione peroxidases in different stages of carcinogenesis." Biochimica et Biophysica Acta (BBA) - General Subjects **1790**(11): 1555-1568.

Burk RF and Hill KE (2005). "Selenoprotein P: An extracellular protein with unique physical characteristics and a role in selenium homeostasis." Annual Review of Nutrition **25**(1): 215-235.

Burk RF, Hill KE, Nakayama A, Mostert V, Levander XA, Motley AK, Johnson DA, Johnson JA, Freeman ML and Austin LM (2008). "Selenium deficiency activates mouse liver Nrf2-ARE but vitamin E deficiency does not." Free Radical Biology and Medicine **44**(8): 1617.

Campos AC, Molognoni F, Melo FH, Galdieri LC, Carneiro CR, D'Almeida V, Correa M and Jasiulionis MG (2007). "Oxidative stress modulates

DNA methylation during melanocyte anchorage blockade associated with malignant transformation." Neoplasia **9**(12): 1111-21.

Cassidy PB, Edes K, Nelson CC, Parsawar K, Fitzpatrick FA and Moos PJ (2006). "Thioredoxin reductase is required for the inactivation of tumor suppressor p53 and for apoptosis induced by endogenous electrophiles." Carcinogenesis **27**(12): 2538-2549.

Cedar H and Bergman Y (2009). "Linking DNA methylation and histone modification: patterns and paradigms." Nat Rev Genet **10**(5): 295-304.

Cerda S and Weitzman SA (1997). "Influence of oxygen radical injury on DNA methylation." Mutation Research/Reviews in Mutation Research **386**(2): 141.

Chadwick RW, George SE and Claxton LD (1992). "Role of the gastrointestinal mucosa and microflora in the bioactivation of dietary and environmental mutagens or carcinogens." Drug Metab Rev **24**(4): 425-92.

Chalitchagorn K, Shuangshoti S, Hourpai N, Kongruttanachok N, Tangkijvanich P, Thong-ngam D, Voravud N, Sriuranpong V and Mutirangura A (2004). "Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis." Oncogene **23**(54): 8841-6.

Chang YF, Imam JS and Wilkinson MF (2007). "The nonsense-mediated decay RNA surveillance pathway." Annu Rev Biochem **76**: 51-74.

Chen J, Rocken C, Lofton-Day C, Schulz H-U, Muller O, Kutzner N, Malfertheiner P and Ebert MPA (2005). "Molecular analysis of APC promoter methylation and protein expression in colorectal cancer metastasis." Carcinogenesis **26**(1): 37-43.

Chen YR, Wang W, Kong AN and Tan TH (1998). "Molecular mechanisms of c-Jun N-terminal kinase-mediated apoptosis induced by anticarcinogenic isothiocyanates." J Biol Chem **273**(3): 1769-75.

Chiao JW, Chung FL, Kancherla R, Ahmed T, Mittelman A and Conaway CC (2002). "Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells." Int J Oncol **20**(3): 631-6.

Choi SW, Friso S, Keyes MK and Mason JB (2005). "Folate supplementation increases genomic DNA methylation in the liver of elder rats." British Journal of Nutrition **93**(1): 31.

Chu F-F, Esworthy RS, Chu PG, Longmate JA, Huycke MM, Wilczynski S and Doroshow JH (2004). "Bacteria-Induced Intestinal Cancer in Mice with Disrupted Gpx1 and Gpx2 Genes." Cancer Res **64**(3): 962-968.

Chu F-F, Esworthy RS and Doroshow JH (2004). "Role of Se-dependent glutathione peroxidases in gastrointestinal inflammation and cancer." Free Radical Biology and Medicine **36**(12): 1481.

Chu F-F, Esworthy RS, Lee L and Wilczynski S (1999). "Retinoic Acid Induces Gpx2 Gene Expression in MCF-7 Human Breast Cancer Cells." J. Nutr. **129**(10): 1846-1854.

Chung F-L, Conaway CC, Rao CV and Reddy BS (2000). "Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate." Carcinogenesis **21**(12): 2287-2291.

Clark LC, Combs GF, Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham GF and Gross EG (1996). "Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group." JAMA **276**(24): 1957-1963.

Clarke JD, Dashwood RH and Ho E (2008). "Multi-targeted prevention of cancer by sulforaphane." Cancer Letters **269**(2): 291-304.

Colotta F, Allavena P, Sica A, Garlanda C and Mantovani A (2009). "Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability." Carcinogenesis **30**(7): 1073-81.

Combs GJ and Lu J (2006). Selenium as a cancer preventive agent. In: Selenium: Its Molecular Biology and Role in Human Health. Hatfield D. L., M. J. Berry and V. N. Gladyshev. Boston, MA, Springer Science+Business Media, LLC.

Conaway CC, Getahun SM, Liebes LL, Pusateri DJ, Topham DK, Botero-Omary M and Chung FL (2000). "Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli." Nutr Cancer **38**(2): 168-78.

Corella D, Cortina P, Guillen M and Gonzalez JI (1996). "Dietary habits and geographic variation in stomach cancer mortality in Spain." Eur J Cancer Prev **5**(4): 249-57.

Cox R (1985). "Selenite: a good inhibitor of rat-liver DNA methylase." Biochem Int **10**(1): 63-9.

Cox R and Goorha S (1986). "A study of the mechanism of selenite-induced hypomethylated DNA and differentiation of Friend erythroleukemic cells." Carcinogenesis **7**(12): 2015-2018.

Cravo ML, Mason JB, Dayal Y, Hutchinson M, Smith D, Selhub J and Rosenberg IH (1992). "Folate Deficiency Enhances the Development of Colonic Neoplasia in Dimethylhydrazine-treated Rats." Cancer Res **52**(18): 5002-5006.

Crosley LK, Méplan C, Nicol F, Rundlöf AK, Arnér ESJ, Hesketh JE and Arthur JR (2007). "Differential regulation of expression of cytosolic and mitochondrial thioredoxin reductase in rat liver and kidney." Archives of Biochemistry and Biophysics **459**(2): 178-188.

Cuello S, Ramos S, Mateos R, Martin MA, Madrid Y, Camara C, Bravo L and Goya L (2007). "Selenium methylselenocysteine protects human hepatoma HepG2 cells against oxidative stress induced by tert-butyl hydroperoxide." Anal Bioanal Chem **389**(7-8): 2167-78.

Curtis RK, Oresic M and Vidal-Puig A (2005). "Pathways to the analysis of microarray data." Trends in Biotechnology **23**(8): 429-435.

Dashwood R and Ho E (2008). "Dietary agents as histone deacetylase inhibitors: sulforaphane and structurally related isothiocyanates." Nutrition Reviews **66**(s1): S36-S38.

Davis CD and Milner JA (2007). "Biomarkers for diet and cancer prevention research: potentials and challenges." Acta Pharmacologica Sinica **28**(9): 1262-1273.

Davis CD and Uthus EO (2002). "Dietary Selenite and Azadeoxycytidine Treatments Affect Dimethylhydrazine-Induced Aberrant Crypt Formation in Rat Colon and DNA Methylation in HT-29 Cells." J. Nutr. **132**(2): 292-297.

Davis CD and Uthus EO (2004). "DNA Methylation, Cancer Susceptibility, and Nutrient Interactions." Experimental Biology and Medicine **229**(10): 988-995.

Davis CD, Uthus EO and Finley JW (2000). "Dietary Selenium and Arsenic Affect DNA Methylation In Vitro in Caco-2 Cells and In Vivo in Rat Liver and Colon." J. Nutr. **130**(12): 2903-2909.

Davis CD, Zeng H and Finley JW (2002). "Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice." J Nutr **132**(2): 307-9.

Deng G, Song G-A, Pong E, Sleisenger M and Kim YS (2004). "Promoter Methylation Inhibits APC Gene Expression by Causing Changes in Chromatin Conformation and Interfering with the Binding of Transcription Factor CCAAT-Binding Factor." Cancer Res **64**(8): 2692-2698.

Dinkova-Kostova AT and Talalay P (2008). "Direct and indirect antioxidant properties of inducers of cytoprotective proteins." Mol Nutr Food Res **52 Suppl 1**: S128-38.

Dong Y, Lisk D, Block E and Ip C (2001). "Characterization of the biological activity of gamma-glutamyl-Se-methylselenocysteine: A novel,

naturally occurring anticancer agent from garlic." Cancer Res **61**(7): 2923-2928.

Down TA, Rakyan VK, Turner DJ, Flicek P, Li H, Kulesha E, Graf S, Johnson N, Herrero J, Tomazou EM, Thorne NP, Backdahl L, Herberth M, Howe KL, Jackson DK, Miretti MM, Marioni JC, Birney E, Hubbard TJP, Durbin R, Tavare S and Beck S (2008). "A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis." Nat Biotech **26**(7): 779-785.

Drake EN (2006). "Cancer chemoprevention: Selenium as a prooxidant, not an antioxidant." Medical Hypotheses **67**(2): 318-322.

Druesne-Pecollo N, Chaumontet C, Pagniez A, Vaugelade P, Bruneau A, Thomas M, Cherbuy C, Duée P-H and Martel P (2007). "In vivo treatment by diallyl disulfide increases histone acetylation in rat colonocytes." Biochemical and Biophysical Research Communications **354**(1): 140-147.

Druesne N, Pagniez A, Mayeur C, Thomas M, Cherbuy C, Duee P-H, Martel P and Chaumontet C (2004). "Diallyl disulfide (DADS) increases histone acetylation and p21waf1/cip1 expression in human colon tumor cell lines." Carcinogenesis **25**(7): 1227-1236.

Duthie SJ (2010). "Folate and cancer: how DNA damage, repair and methylation impact on colon carcinogenesis." J Inherit Metab Dis.

Duthie SJ, Collins AR, Duthie GG and Dobson VL (1997). "Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes." Mutat Res **393**(3): 223-31.

Duthie SJ, Narayanan S, Blum S, Pirie L and Brand GM (2000). "Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells." Nutr Cancer **37**(2): 245-51.

Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV and Laird PW (2000). "MethyLight: a high-throughput assay to measure DNA methylation." Nucl. Acids Res. **28**(8): e32-.

Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV and Laird PW (1999). "CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression." Cancer Res **59**(10): 2302-6.

Edler L and Kopp-Schneider A (2005). "Origins of the mutational origin of cancer." Int. J. Epidemiol. **34**(5): 1168-1170.

Egger G, Liang G, Aparicio A and Jones PA (2004). "Epigenetics in human disease and prospects for epigenetic therapy." Nature **429**(6990): 457-463.

Eggler AL, Gay KA and Mesecar AD (2008). "Molecular mechanisms of natural products in chemoprevention: Induction of cytoprotective enzymes by Nrf2." Molecular Nutrition & Food Research **52**(S1): 84-94.

Elahi A, Li Z, Timothy JY, Sigal G, Said S and David S (2008). "HPP1 mediated tumor suppression requires activation of STAT1 pathways." International Journal of Cancer **122**(7): 1567-1572.

Estecio MR and Issa JP (2009). "Tackling the methylome: recent methodological advances in genome-wide methylation profiling." Genome Med **1**(11): 106.

Esteller M (2005). Impact of DNA methylation on health and disease. In: DNA methylation: approaches, methods, and applications. Esteller M. Boca Raton, Fla; London, CRC**:** 1-9 p.

Esteller M (2007). "Cancer epigenomics: DNA methylomes and histone-modification maps." Nat Rev Genet **8**(4): 286-298.

Esteller M, Corn PG, Baylin SB and Herman JG (2001). "A Gene Hypermethylation Profile of Human Cancer." Cancer Res **61**(8): 3225-3229.

Esteller M, Sparks A, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Gonzalez S, Tarafa G, Sidransky D, Meltzer SJ, Baylin SB and Herman JG (2000). "Analysis of Adenomatous Polyposis Coli Promoter Hypermethylation in Human Cancer." Cancer Res **60**(16): 4366-4371.

Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB and Herman JG (2000). "Hypermethylation-associated Inactivation of p14ARF Is Independent of p16INK4a Methylation and p53 Mutational Status." Cancer Res **60**(1): 129-133.

Esworthy RS, Aranda R, Martin MG, Doroshow JH, Binder SW and Chu F-F (2001). "Mice with combined disruption of Gpx1 and Gpx2 genes have colitis." Am J Physiol Gastrointest Liver Physiol **281**(3): G848-855.

Esworthy RS, Yang L, Frankel PH and Chu F-F (2005). "Epithelium-Specific Glutathione Peroxidase, Gpx2, Is Involved in the Prevention of Intestinal Inflammation in Selenium-Deficient Mice." J. Nutr. **135**(4): 740-745.

Evan GI and Vousden KH (2001). "Proliferation, cell cycle and apoptosis in cancer." Nature **411**(6835): 342-8.

Fahey JW, Zalcmann AT and Talalay P (2001). "The chemical diversity and distribution of glucosinolates and isothiocyanates among plants." Phytochemistry **56**(1): 5-51.

Fairweather-Tait S, Bao Y, Broadley M, Collings R, Ford D, Hesketh J and Hurst R (2010). "Selenium in Human Health and Disease." Antioxid Redox Signal.

Fairweather-Tait SJ, Collings R and Hurst R (2010). "Selenium bioavailability: current knowledge and future research requirements." Am J Clin Nutr **91**(5): 1484S-1491S.

Fang JY and Xiao SD (2003). "Folic acid, polymorphism of methyl-group metabolism genes, and DNA methylation in relation to GI carcinogenesis." J Gastroenterol **38**(9): 821-9.

Fang M, Chen D and Yang CS (2007). "Dietary Polyphenols May Affect DNA Methylation." J. Nutr. **137**(1): 223S-228.

Fang MZ, Chen D, Sun Y, Jin Z, Christman JK and Yang CS (2005). "Reversal of Hypermethylation and Reactivation of p16INK4a, RARβ, and MGMT Genes by Genistein and Other Isoflavones from Soy." Clin Cancer Res **11**(19): 7033-7041.

Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W and Yang CS (2003). "Tea Polyphenol (-)-Epigallocatechin-3-Gallate Inhibits DNA Methyltransferase and Reactivates Methylation-Silenced Genes in Cancer Cell Lines." Cancer Res **63**(22): 7563-7570.

Faulkner K, Mithen R and Williamson G (1998). "Selective increase of the potential anticarcinogen 4-methylsulphinylbutyl glucosinolate in broccoli." Carcinogenesis **19**(4): 605-9.

Fearon ER and Vogelstein B (1990). "A genetic model for colorectal tumorigenesis." Cell **61**(5): 759-767.

Feltus FA, Lee EK, Costello JF, Plass C and Vertino PM (2003). "Predicting aberrant CpG island methylation." Proceedings of the National Academy of Sciences of the United States of America **100**(21): 12253-12258.

Fiala ES, Staretz ME, Pandya GA, El-Bayoumy K and Hamilton SR (1998). "Inhibition of DNA cytosine methyltransferase by chemopreventive selenium compounds, determined by an improved assay for DNA cytosine methyltransferase and DNA cytosine methylation." Carcinogenesis **19**(4): 597-604.

Finley JW (2003). "Reduction of cancer risk by consumption of selenium-enriched plants: enrichment of broccoli with selenium increases the anticarcinogenic properties of broccoli." J Med Food **6**(1): 19-26.

Finley JW and Davis CD (2001). "Selenium (Se) from high-selenium broccoli is utilized differently than selenite, selenate and selenomethionine, but is more effective in inhibiting colon carcinogenesis." Biofactors **14**(1-4): 191-6.

Finley JW, Davis CD and Feng Y (2000). "Selenium from high selenium broccoli protects rats from colon cancer." J Nutr **130**(9): 2384-9.

Finley JW, Ip C, Lisk DJ, Davis CD, Hintze KJ and Whanger PD (2001). "Cancer-protective properties of high-selenium broccoli." J Agric Food Chem **49**(5): 2679-83.

Firestein R, Bass AJ, Kim SY, Dunn IF, Silver SJ, Guney I, Freed E, Ligon AH, Vena N, Ogino S, Chheda MG, Tamayo P, Finn S, Shrestha Y, Boehm JS, Jain S, Bojarski E, Mermel C, Barretina J, Chan JA, Baselga J, Tabernero J, Root DE, Fuchs CS, Loda M, Shivdasani RA, Meyerson M and Hahn WC (2008). "CDK8 is a colorectal cancer oncogene that regulates [bgr]-catenin activity." Nature **455**(7212): 547-551.

Flood A, Caprario L, Chaterjee N, Lacey JV, Jr., Schairer C and Schatzkin A (2002). "Folate, methionine, alcohol, and colorectal cancer in a prospective study of women in the United States." Cancer Causes Control **13**(6): 551-61.

Florian S, Wingler K, Schmehl K, Jacobasch G, Kreuzer OJ, Meyerhof W and Brigelius-Flohe R (2001). "Cellular and subcellular localization of gastrointestinal glutathione peroxidase in normal and malignant human intestinal tissue." Free Radic Res **35**(6): 655-63.

Fogh J and Orfeo T (1977). "One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice." J Natl Cancer Inst **59**(1): 221-6.

Fu Y, Sies H and Lei XG (2001). "Opposite roles of selenium-dependent glutathione peroxidase-1 in superoxide generator diquat- and peroxynitrite-induced apoptosis and signaling." J Biol Chem **276**(46): 43004-9.

Gallegos A, Berggren M, Gasdaska JR and Powis G (1997). "Mechanisms of the Regulation of Thioredoxin Reductase Activity in Cancer Cells by the Chemopreventive Agent Selenium." Cancer Res **57**(21): 4965-4970.

Gamet-Payrastre L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, Gasc N, Tulliez J and Terce F (2000). "Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells." Cancer Res **60**(5): 1426-33.

Ganther HE (1999). "Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase." Carcinogenesis **20**(9): 1657-1666.

Gasper AV, Traka M, Bacon JR, Smith JA, Taylor MA, Hawkey CJ, Barrett DA and Mithen RF (2007). "Consuming broccoli does not induce genes associated with xenobiotic metabolism and cell cycle control in human gastric mucosa." J Nutr **137**(7): 1718-24.

Geenen RWF, Hussain SM, Cademartiri F, Poley J-W, Siersema PD and Krestin GP (2003). "CT and MR Colonography: Scanning Techniques, Postprocessing, and Emphasis on Polyp Detection." Radiographics: e18.

Giovannucci E, Stampfer MJ, Colditz GA, Rimm EB, Trichopoulos D, Rosner BA, Speizer FE and Willett WC (1993). "Folate, methionine, and alcohol intake and risk of colorectal adenoma." J Natl Cancer Inst **85**(11): 875-84.

Gladyshev VN, Factor VM, Housseau F and Hatfield DL (1998). "Contrasting patterns of regulation of the antioxidant selenoproteins, thioredoxin reductase, and glutathione peroxidase, in cancer cells." Biochem Biophys Res Commun **251**(2): 488-93.

Goya L, Martin M, Ramos S, Mateos R and Bravo L (2009). "A Cell Culture Model for the Assessment of the Chemopreventive Potential of Dietary Compounds." Current Nutrition & Food Science **5**(1): 56-64(9).

Greenblatt MS, Bennett WP, Hollstein M and Harris CC (1994). "Mutations in the p53 Tumor Suppressor Gene: Clues to Cancer Etiology and Molecular Pathogenesis." Cancer Res **54**(18): 4855-4878.

Hanausek M, Walaszek Z and Slaga TJ (2004). Redox modulation in tumor initiation, promotion, and progression. In: redox-genome interactions in health and disease. Fuchs J., M. Podda and L. Packer. USA, Marcel Dekker**:** pp 193-233.

Hardwick JC, Kodach LL, Offerhaus GJ and van den Brink GR (2008). "Bone morphogenetic protein signalling in colorectal cancer." Nat Rev Cancer **8**(10): 806-812.

Harnack L, Jacobs DR, Jr., Nicodemus K, Lazovich D, Anderson K and Folsom AR (2002). "Relationship of folate, vitamin B-6, vitamin B-12, and methionine intake to incidence of colorectal cancers." Nutr Cancer **43**(2): 152-8.

Harris KE and Jeffery EH (2008). "Sulforaphane and erucin increase MRP1 and MRP2 in human carcinoma cell lines." J Nutr Biochem **19**(4): 246-54.

Hashimoto K, Kokubun S, Itoi E and Roach H (2007). "Improved quantification of DNA methylation using methylation-sensitive restriction enzymes and real-time PCR." Epigenetics **2**(2): 86-95.

Hatfield DL (2001). Selenium: its molecular biology and role in human health, Kluwer Academic Pub.

Hatfield DL, Berry MJ and Gladyshev VN (2006). Selenium: Its Molecular Biology and Role in Human Health. Boston, MA, Springer Science+Business Media, LLC.

Hecht SS (1999). "Chemoprevention of Cancer by Isothiocyanates, Modifiers of Carcinogen Metabolism." J. Nutr. **129**(3): 768-.

Hee KS, Bae SI, Lee HS and Kim WH (2003). "Alteration of O6-methylguanine-DNA methyltransferase in colorectal neoplasms in sporadic and familial adenomatous polyposis patients." Molecular Carcinogenesis **37**(1): 32-38.

Herman JG and Baylin SB (2003). "Gene Silencing in Cancer in Association with Promoter Hypermethylation." N Engl J Med **349**(21): 2042-2054.

Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB (1996). "Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands." Proceedings of the National Academy of Sciences **93**(18): 9821-9826.

Herr I and Büchler MW (2010). "Dietary constituents of broccoli and other cruciferous vegetables: Implications for prevention and therapy of cancer." Cancer Treatment Reviews **36**(5): 377-383.

Hesketh JE and Villette S (2002). "Intracellular trafficking of micronutrients: from gene regulation to nutrient requirements." Proc Nutr Soc **61**(4): 405-14.

Hintze KJ, Keck A-S, Finley JW and Jeffery EH (2003). "Induction of hepatic thioredoxin reductase activity by sulforaphane, both in Hepa1c1c7 cells and in male Fisher 344 rats." The Journal of Nutritional Biochemistry **14**(3): 173-179.

Hintze KJ, Wald KA, Zeng H, Jeffery EH and Finley JW (2003). "Thioredoxin reductase in human hepatoma cells is transcriptionally regulated by sulforaphane and other electrophiles via an antioxidant response element." J Nutr **133**(9): 2721-7.

Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K and Yodoi J (1997). "AP-1 transcriptional activity is regulated by a direct association between thioredoxin andâ€‰Ref-1." Proceedings of the National Academy of Sciences of the United States of America **94**(8): 3633-3638.

Ho E, Clarke JD and Dashwood RH (2009). "Dietary Sulforaphane, a Histone Deacetylase Inhibitor for Cancer Prevention." J. Nutr. **139**(12): 2393-2396.

Holmberg L, Ohlander EM, Byers T, Zack M, Wolk A, Bruce A, Bergstrom R, Bergkvist L and Adami HO (1996). "A search for recall bias in a case-control study of diet and breast cancer." Int J Epidemiol **25**(2): 235-44.

Holst B and Williamson G (2004). "A critical review of the bioavailability of glucosinolates and related compounds." Nat Prod Rep **21**(3): 425-47.

Hu R, Khor TO, Shen G, Jeong W-S, Hebbar V, Chen C, Xu C, Reddy B, Chada K and Kong A-NT (2006). "Cancer chemoprevention of intestinal polyposis in ApcMin/+ mice by sulforaphane, a natural product derived from cruciferous vegetable." Carcinogenesis **27**(10): 2038-2046.

Hu R, Kim BR, Chen C, Hebbar V and Kong AN (2003). "The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells." Carcinogenesis **24**(8): 1361-7.

Hu Y, Benya RV, Carroll RE and Diamond AM (2005). "Allelic Loss of the Gene for the GPX1 Selenium-Containing Protein Is a Common Event in Cancer." J. Nutr. **135**(12): 3021S-3024.

Humphries A and Wright NA (2008). "Colonic crypt organization and tumorigenesis." Nat Rev Cancer **8**(6): 415-24.

Hurst R, Armah CN, Dainty JR, Hart DJ, Teucher B, Goldson AJ, Broadley MR, Motley AK and Fairweather-Tait SJ (2010). "Establishing optimal selenium status: results of a randomized, double-blind, placebo-controlled trial." Am J Clin Nutr **91**(4): 923-31.

Hwang C, Sinskey AJ and Lodish HF (1992). "Oxidized redox state of glutathione in the endoplasmic reticulum." Science **257**(5076): 1496-502.

Iacopetta B, Grieu F, Phillips M, Ruszkiewicz A, Moore J, Minamoto T and Kawakami K (2007). "Methylation levels of LINE-1 repeats and CpG island loci are inversely related in normal colonic mucosa." Cancer Sci **98**(9): 1454-60.

IARC/WHO (2004). Cruciferous vegetables, isothiocyanates and indoles. Lyon, IARC Press.

Ip C (1998). "Lessons from Basic Research in Selenium and Cancer Prevention." J. Nutr. **128**(11): 1845-1854.

Ip C, Birringer M, Block E, Kotrebai M, Tyson JF, Uden PC and Lisk DJ (2000). "Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention." J Agric Food Chem **48**(9): 4452.

Ip C, Dong Y and Ganther HE (2002). "New concepts in selenium chemoprevention." Cancer Metastasis Rev **21**(3-4): 281-9.

Ip C, Hayes C, Budnick RM and Ganther HE (1991). "Chemical Form of Selenium, Critical Metabolites, and Cancer Prevention." Cancer Res **51**(2): 595-600.

Ip C, Thompson HJ, Zhu Z and Ganther HE (2000). "In Vitro and in Vivo Studies of Methylseleninic Acid: Evidence That a Monomethylated Selenium Metabolite Is Critical for Cancer Chemoprevention." Cancer Res **60**(11): 2882-2886.

Irizarry RA, Ladd-Acosta C, Carvalho B, Wu H, Brandenburg SA, Jeddeloh JA, Wen B and Feinberg AP (2008). "Comprehensive high-throughput arrays for relative methylation (CHARM)." Genome Res. **18**(5): 780-790.

Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S and Feinberg AP (2009). "The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores." Nat Genet **41**(2): 178-186.

Irons R, Carlson BA, Hatfield DL and Davis CD (2006). "Both Selenoproteins and Low Molecular Weight Selenocompounds Reduce Colon Cancer Risk in Mice with Genetically Impaired Selenoprotein Expression." J. Nutr. **136**(5): 1311-1317.

Irons R, Tsuji PA, Carlson BA, Ouyang P, Yoo M-H, Xu X-M, Hatfield DL, Gladyshev VN and Davis CD (2010). "Deficiency in the 15-kDa Selenoprotein Inhibits Tumorigenicity and Metastasis of Colon Cancer Cells." Cancer Prevention Research **3**(5): 630-639.

Issa J-PJ, Ottaviano YL, Celano P, Hamilton SR, Davidson NE and Baylin SB (1994). "Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon." Nat Genet **7**(4): 536-540.

Jablonska E, Gromadzinska J, Sobala W, Reszka E and Wasowicz W (2008). "Lung cancer risk associated with selenium status is modified in smoking individuals by Sep15 polymorphism." Eur J Nutr **47**(1): 47-54.

Jackson MI and Combs Jr GF (2008). "Selenium and anticarcinogenesis: underlying mechanisms." Curr Opin Clin Nutr Metab Care **11**(6): 718-26.

Jacobs ET, Jiang R, Alberts DS, Greenberg ER, Gunter EW, Karagas MR, Lanza E, Ratnasinghe L, Reid ME, Schatzkin A, Smith-Warner SA, Wallace K and Martinez ME (2004). "Selenium and colorectal adenoma: results of a pooled analysis." J Natl Cancer Inst **96**(22): 1669-75.

Jakubikova J, Bao Y, Bodo J and Sedlak J (2006). "Isothiocyanate iberin modulates phase II enzymes, posttranslational modification of histones and inhibits growth of Caco-2 cells by inducing apoptosis." Neoplasma **53**(6): 463-70.

Jakubikova J, Bao Y and Sedlak J (2005). "Isothiocyanates induce cell cycle arrest, apoptosis and mitochondrial potential depolarization in HL-60 and multidrug-resistant cell lines." Anticancer Res **25**(5): 3375-86.

Jakubikova J, Sedlak J, Bacon J, Goldson A and Bao Y (2005). "Effects of MEK1 and PI3K inhibitors on allyl-, benzyl- and phenylethyl-isothiocyanate-induced G2/M arrest and cell death in Caco-2 cells." Int J Oncol **27**(5): 1449-58.

Jakubikova J, Sedlak J, Bod'o J and Bao Y (2006). "Effect of Isothiocyanates on Nuclear Accumulation of NF-KB, Nrf2, and Thioredoxin in Caco-2 Cells." J. Agric. Food Chem. **54**(5): 1656-1662.

Jeong WS, Jun M and Kong ANT (2006). "Nrf2: A Potential Molecular Target for Cancer Chemoprevention by Natural Compounds." Antioxidants & Redox Signaling **8**(1-2): 99-106.

Ji Y and Morris ME (2003). "Determination of phenethyl isothiocyanate in human plasma and urine by ammonia derivatization and liquid chromatography-tandem mass spectrometry." Anal Biochem **323**(1): 39-47.

Jiang Z-Q, Chen C, Yang B, Hebbar V and Kong ANT (2003). "Differential responses from seven mammalian cell lines to the treatments of detoxifying enzyme inducers." Life Sciences **72**(20): 2243-2253.

Jiao D, Eklind KI, Choi CI, Desai DH, Amin SG and Chung FL (1994). "Structure-activity relationships of isothiocyanates as mechanism-based inhibitors of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in A/J mice." Cancer Res **54**(16): 4327-33.

Johnson IT (2002). "Glucosinolates: bioavailability and importance to health." Int J Vitam Nutr Res **72**(1): 26-31.

Johnson IT and Belshaw NJ (2008). "Environment, diet and CpG island methylation: Epigenetic signals in gastrointestinal neoplasia." Food and Chemical Toxicology **46**(4): 1346-1359.

Johnson IT, Williamson G and Musk SRR (1994). "Anticarcinogenic Factors in Plant Foods: A New Class of Nutrients?" Nutrition Research Reviews **7**(01): 175-204.

Jones PA and Baylin SB (2007). "The epigenomics of cancer." Cell **128**(4): 683-92.

Jones PA and Liang G (2009). "Rethinking how DNA methylation patterns are maintained." Nature Reviews Genetics **10**(11): 805-811.

Juge N, Mithen RF and Traka M (2007). "Molecular basis for chemoprevention by sulforaphane: a comprehensive review." Cell Mol Life Sci **64**(9): 1105-27.

Jung U, Zheng X, Yoon S-O and Chung A-S (2001). "Se-Methylselenocysteine induces apoptosis mediated by reactive oxygen species in HL-60 cells." Free Radical Biology and Medicine **31**(4): 479.

Juttermann R, Li E and Jaenisch R (1994). "Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation." Proceedings of the National Academy of Sciences of the United States of America **91**(25): 11797-11801.

Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, Jessup JM and Kolodner R (1997). "Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines." Cancer Res **57**(5): 808-11.

Kang GH, Lee HJ, Hwang KS, Lee S, Kim J-H and Kim J-S (2003). "Aberrant CpG Island Hypermethylation of Chronic Gastritis, in Relation to Aging, Gender, Intestinal Metaplasia, and Chronic Inflammation." Am J Pathol **163**(4): 1551-1556.

Kang KW, Lee SJ, Park JW and Kim SG (2002). "Phosphatidylinositol 3-Kinase Regulates Nuclear Translocation of NF-E2-Related Factor 2 through Actin Rearrangement in Response to Oxidative Stress." Molecular Pharmacology **62**(5): 1001-1010.

Keck AS and Finley JW (2004). "Cruciferous vegetables: cancer protective mechanisms of glucosinolate hydrolysis products and selenium." Integr Cancer Ther **3**(1): 5-12.

Kerr D (2003). "Clinical development of gene therapy for colorectal cancer." Nat Rev Cancer **3**(8): 615-622.

Keum YS, Owuor ED, Kim BR, Hu R and Kong AN (2003). "Involvement of Nrf2 and JNK1 in the activation of antioxidant responsive element

(ARE) by chemopreventive agent phenethyl isothiocyanate (PEITC)." Pharm Res **20**(9): 1351-6.

Keum YS, Yu S, Chang PP, Yuan X, Kim JH, Xu C, Han J, Agarwal A and Kong AN (2006). "Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells." Cancer Res **66**(17): 8804-13.

Khulan B, Thompson RF, Ye K, Fazzari MJ, Suzuki M, Stasiek E, Figueroa ME, Glass JL, Chen Q, Montagna C, Hatchwell E, Selzer RR, Richmond TA, Green RD, Melnick A and Greally JM (2006). "Comparative isoschizomer profiling of cytosine methylation: the HELP assay." Genome Res **16**(8): 1046-55.

Kim B-R, Hu R, Keum Y-S, Hebbar V, Shen G, Nair SS and Kong ANT (2003). "Effects of Glutathione on Antioxidant Response Element-Mediated Gene Expression and Apoptosis Elicited by Sulforaphane." Cancer Res **63**(21): 7520-7525.

Kim EH, Sohn S, Kwon HJ, Kim SU, Kim M-J, Lee S-J and Choi KS (2007). "Sodium Selenite Induces Superoxide-Mediated Mitochondrial Damage and Subsequent Autophagic Cell Death in Malignant Glioma Cells." Cancer Research **67**(13): 6314-6324.

Kim T, Jung U, Cho D-Y and Chung A-S (2001). "Se-Methylselenocysteine induces apoptosis through caspase activation in HL-60 cells." Carcinogenesis **22**(4): 559-565.

Kim YI, Salomon RN, Graeme-Cook F, Choi SW, Smith DE, Dallal GE and Mason JB (1996). "Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats." Gut **39**(5): 732-40.

Kleivi K, Teixeira MR, Eknaes M, Diep CB, Jakobsen KS, Hamelin R and Lothe RA (2004). "Genome signatures of colon carcinoma cell lines." Cancer Genetics and Cytogenetics **155**(2): 119-131.

Králová V, Červinka M and Rudolf E (2010). "ROS mediate selenite-induced apoptosis in colon cancer cells." Central European Journal of Biology **5**(2): 166-177.

Kruhoffer M, Jensen JL, Laiho P, Dyrskjot L, Salovaara R, Arango D, Birkenkamp-Demtroder K, Sorensen FB, Christensen LL, Buhl L, Mecklin JP, Jarvinen H, Thykjaer T, Wikman FP, Bech-Knudsen F, Juhola M, Nupponen NN, Laurberg S, Andersen CL, Aaltonen LA and Orntoft TF (2005). "Gene expression signatures for colorectal cancer microsatellite status and HNPCC." Br J Cancer **92**(12): 2240-8.

Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigo R and Gladyshev VN (2003). "Characterization of Mammalian Selenoproteomes." Science **300**(5624): 1439-1443.

Kumar A and Sabbioni G (2010). "New Biomarkers for Monitoring the Levels of Isothiocyanates in Humans." Chemical Research in Toxicology **23**(4): 756-765.

Kumar A, Vineis P, Sacerdote C, Fiorini L and Sabbioni G (2010). "Determination of new biomarkers to monitor the dietary consumption of isothiocyanates." Biomarkers.

Kumar Kundu J, Na H-K and Surh Y-J (2009). Intracellular signaling molecules as targets of selected dietary chemopreventive agents. In: Dietary Modulation of Cell Signaling Pathways Surh Y.-J. Boca Raton, CRC Press**:** p. 1-33.

Laird PW (2003). "The power and the promise of DNA methylation markers." Nature Reviews Cancer **3**: 253-266.

Laird PW (2010). "Principles and challenges of genome-wide DNA methylation analysis." Nat Rev Genet **11**(3): 191-203.

Laitinen L (2006). "Caco-2 cell cultures in the assessment of intestinal absorption: Effects of some co-administered drugs and natural compounds in biological matrices." Academic Dissertation: 1-66.

Lamprecht SA and Lipkin M (2003). "Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms." Nat Rev Cancer **3**(8): 601-14.

Lau A, Villeneuve NF, Sun Z, Wong PK and Zhang DD (2008). "Dual roles of Nrf2 in cancer." Pharmacological Research **58**(5-6): 262-270.

Lee WJ, Shim J-Y and Zhu BT (2005). "Mechanisms for the Inhibition of DNA Methyltransferases by Tea Catechins and Bioflavonoids." Mol Pharmacol **68**(4): 1018-1030.

Lee WJ and Zhu BT (2006). "Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols." Carcinogenesis **27**(2): 269-277.

Li D, Wu K, Howie AF, Beckett GJ, Wang W and Bao Y (2008). "Synergy between broccoli sprout extract and selenium in the upregulation of thioredoxin reductase in human hepatocytes." Food Chemistry **110**(1): 193.

Li H, Stampfer MJ, Giovannucci EL, Morris JS, Willett WC, Gaziano JM and Ma J (2004). "A Prospective Study of Plasma Selenium Levels and Prostate Cancer Risk." J. Natl. Cancer Inst. **96**(9): 696-703.

Li J, Johnson D, Calkins M, Wright L, Svendsen C and Johnson J (2005). "Stabilization of Nrf2 by tBHQ Confers Protection against Oxidative Stress-Induced Cell Death in Human Neural Stem Cells." Toxicol. Sci. **83**(2): 313-328.

Lin HJ, Probst-Hensch NM, Louie AD, Kau IH, Witte JS, Ingles SA, Frankl HD, Lee ER and Haile RW (1998). "Glutathione transferase null genotype, broccoli, and lower prevalence of colorectal adenomas." Cancer Epidemiol Biomarkers Prev **7**(8): 647-52.

Lin YM, Furukawa Y, Tsunoda T, Yue CT, Yang KC and Nakamura Y (2002). "Molecular diagnosis of colorectal tumors by expression profiles of 50 genes expressed differentially in adenomas and carcinomas." Oncogene **21**(26): 4120-8.

Lincoln DT, Ali Emadi EM, Tonissen KF and Clarke FM (2003). "The thioredoxin-thioredoxin reductase system: over-expression in human cancer." Anticancer Res **23**(3B): 2425-33.

Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, Parnes HL, Minasian LM, Gaziano JM, Hartline JA, Parsons JK, Bearden JD, 3rd, Crawford ED, Goodman GE, Claudio J, Winquist E, Cook ED, Karp DD, Walther P, Lieber MM, Kristal AR, Darke AK, Arnold KB, Ganz PA, Santella RM, Albanes D, Taylor PR, Probstfield JL, Jagpal TJ, Crowley JJ, Meyskens FL, Jr., Baker LH and Coltman CA, Jr. (2009). "Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT)." JAMA **301**(1): 39-51.

Liu RH (2003). "Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals." Am J Clin Nutr **78**(3): 517S-520.

Liu RH and Finley J (2005). "Potential Cell Culture Models for Antioxidant Research." Journal of Agricultural and Food Chemistry **53**(10): 4311-4314.

Livak KJ and Schmittgen TD (2001). "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- ??CT Method." Methods **25**(4): 402-408.

Loeb LA (2001). "A Mutator Phenotype in Cancer." Cancer Res **61**(8): 3230-3239.

London SJ, Yuan J-M, Chung F-L, Gao Y-T, Coetzee GA, Ross RK and Yu MC (2000). "Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China." The Lancet **356**(9231): 724-729.

Lopez-Serra L and Esteller M (2008). "Proteins that bind methylated DNA and human cancer: reading the wrong words." Br J Cancer **98**(12): 1881.

Lu J, Hu H and Jiang C (2009). Regulation of signaling pathways by selenium in cancer. In: Dietary Modulation of Cell Signaling Pathways Surh Y.-J. Boca Raton, CRC Press**:** p. 273-313.

Lu Q, Lin X, Feng J, Zhao X, Gallagher R, Lee MY, Chiao JW and Liu D (2008). "Phenylhexyl isothiocyanate has dual function as histone deacetylase inhibitor and hypomethylating agent and can inhibit myeloma cell growth by targeting critical pathways." Journal of Hematology and Oncology **1**: 6.

Maheo K, Morel F, Langouet S, Kramer H, Le Ferrec E, Ketterer B and Guillouzo A (1997). "Inhibition of Cytochromes P-450 and Induction of Glutathione S-Transferases by Sulforaphane in Primary Human and Rat Hepatocytes." Cancer Res **57**(17): 3649-3652.

Majid S, Dar AA, Ahmad AE, Hirata H, Kawakami K, Shahryari V, Saini S, Tanaka Y, Dahiya AV, Khatri G and Dahiya R (2009). "BTG3 tumor suppressor gene promoter demethylation, histone modification and cell cycle arrest by genistein in renal cancer." Carcinogenesis **30**(4): 662-70.

Malhotra D, Portales-Casamar E, Singh A, Srivastava S, Arenillas D, Happel C, Shyr C, Wakabayashi N, Kensler TW, Wasserman WW and Biswal S (2010). "Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis." Nucleic Acids Res **38**(17): 5718-34.

Mandal PK, Schneider M, Kolle P, Kuhlencordt P, Forster H, Beck H, Bornkamm GW and Conrad M (2010). "Loss of thioredoxin reductase 1 renders tumors highly susceptible to pharmacologic glutathione deprivation." Cancer Res.

Markowitz SD and Bertagnolli MM (2009). "Molecular origins of cancer: Molecular basis of colorectal cancer." N Engl J Med **361**(25): 2449-60.

Martin KR (2006). "Targeting Apoptosis with Dietary Bioactive Agents." Exp. Biol. Med. **231**(2): 117-a-129.

Martinez ME, Marshall JR and Giovannucci E (2008). "Diet and cancer prevention: the roles of observation and experimentation." Nat Rev Cancer **8**(9): 694.

Matthews JR, Wakasugi N, Virelizier JL, Yodoi J and Hay RT (1992). "Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62." Nucleic Acids Res **20**(15): 3821-30.

Matusheski NV and Jeffery EH (2001). "Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile." J Agric Food Chem **49**(12): 5743-9.

Maulik N and Das DK (2008). "Emerging potential of thioredoxin and thioredoxin interacting proteins in various disease conditions." Biochim Biophys Acta **1780**(11): 1368-82.

McDonald SA, Preston SL, Lovell MJ, Wright NA and Jankowski JA (2006). "Mechanisms of disease: from stem cells to colorectal cancer." Nat Clin Pract Gastroenterol Hepatol **3**(5): 267-74.

McPherson MJ and Moller SG (2000). The basic of PCR, Taylor & Francis Group.

Meeran SM, Patel SN and Tollefsbol TO (2010). "Sulforaphane causes epigenetic repression of hTERT expression in human breast cancer cell lines." PLoS One **5**(7): e11457.

Mikol YB, Hoover KL, Creasia D and Poirier LA (1983). "Hepatocarcinogenesis in rats fed methyl-deficient, amino acid-defined diets." Carcinogenesis **4**(12): 1619-1629.

Minucci S and Pelicci PG (2006). "Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer." Nat Rev Cancer **6**(1): 38-51.

Mithen R, Faulkner K, Magrath R, Rose P, Williamson G and Marquez J (2003). "Development of isothiocyanate-enriched broccoli, and its enhanced ability to induce phase 2 detoxification enzymes in mammalian cells." Theor Appl Genet **106**(4): 727-34.

Moos PJ, Edes K, Cassidy P, Massuda E and Fitzpatrick FA (2003). "Electrophilic Prostaglandins and Lipid Aldehydes Repress Redox-sensitive Transcription Factors p53 and Hypoxia-inducible Factor by Impairing the Selenoprotein Thioredoxin Reductase." Journal of Biological Chemistry **278**(2): 745-750.

Mörk H, Al-Taie OH, Bahr K, Zierer A, Beck C, Scheurlen M, Jacob F and Kohrle J (2000). "Inverse mRNA Expression of the Selenocysteine-Containing Proteins GI-GPx and SeP in Colorectal Adenomas Compared With Adjacent Normal Mucosa." Nutrition and Cancer **37**(1): 108 - 116.

Mörk H, Scheurlen M, Al-Taie O, Zierer A, Kraus M, Schöttker K, Jakob F and Köhrle J (2003). "Glutathione peroxidase isoforms as part of the local antioxidative defense system in normal and Barrett's esophagus." International Journal of Cancer **105**(3): 300-304.

Morse MA, Eklind KI, Amin SG, Hecht SS and Chung FL (1989). "Effects of alkyl chain length on the inhibition of NNK-induced lung neoplasia in A/J mice by arylalkyl isothiocyanates." Carcinogenesis **10**(9): 1757-9.

Morse MA, Eklind KI, Hecht SS, Jordan KG, Choi CI, Desai DH, Amin SG and Chung FL (1991). "Structure-activity relationships for inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone lung tumorigenesis by arylalkyl isothiocyanates in A/J mice." Cancer Res **51**(7): 1846-50.

Mund C, Beier V, Bewerunge P, Dahms M, Lyko F and Hoheisel JD (2005). "Array-based analysis of genomic DNA methylation patterns of the tumour suppressor gene p16INK4A promoter in colon carcinoma cell lines." Nucl. Acids Res. **33**(8): e73-.

Munday R and Munday CM (2004). "Induction of Phase II Detoxification Enzymes in Rats by Plant-Derived Isothiocyanates: Comparison of Allyl Isothiocyanate with Sulforaphane and Related Compounds." Journal of Agricultural and Food Chemistry **52**(7): 1867-1871.

Mustacich D and Powis G (2000). "Thioredoxin reductase." Biochem. J. **346**(1): 1-8.

Myohanen SK, Baylin SB and Herman JG (1998). "Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia." Cancer Res **58**(4): 591-3.

Myzak MC, Dashwood WM, Orner GA, Ho E and Dashwood RH (2006). "Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apcmin mice." FASEB J.: 05-4785fje.

Myzak MC, Hardin K, Wang R, Dashwood RH and Ho E (2006). "Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells." Carcinogenesis **27**(4): 811-819.

Myzak MC, Karplus PA, Chung F-L and Dashwood RH (2004). "A Novel Mechanism of Chemoprotection by Sulforaphane: Inhibition of Histone Deacetylase." Cancer Res **64**(16): 5767-5774.

Myzak MC, Tong P, Dashwood W-M, Dashwood RH and Ho E (2007). "Sulforaphane Retards the Growth of Human PC-3 Xenografts and Inhibits HDAC Activity in Human Subjects." Experimental Biology and Medicine **232**(2): 227-234.

Nagasaka T, Koi M, Kloor M, Gebert J, Vilkin A, Nishida N, Shin SK, Sasamoto H, Tanaka N and Matsubara N (2008). "Mutations in both KRAS and BRAF may contribute to the methylator phenotype in colon cancer." Gastroenterology **134**(7): 1950-1960.

Nakamura Y and Miyoshi N (2010). "Electrophiles in foods: the current status of isothiocyanates and their chemical biology." Biosci Biotechnol Biochem **74**(2): 242-55.

Nalvarte I, Damdimopoulos AE, Nystom C, Nordman T, Miranda-Vizuete A, Olsson JM, Eriksson L, Bjornstedt M, Arner ES and Spyrou G (2004). "Overexpression of enzymatically active human cytosolic and mitochondrial thioredoxin reductase in HEK-293 cells. Effect on cell growth and differentiation." J Biol Chem **279**(52): 54510-7.

Nolan T, Hands RE and Bustin SA (2006). "Quantification of mRNA using real-time RT-PCR." Nat Protoc **1**(3): 1559-82.

Nomura AMY, Wilkens LR, Murphy SP, Hankin JH, Henderson BE, Pike MC and Kolonel LN (2008). "Association of vegetable, fruit, and grain intakes with colorectal cancer: the Multiethnic Cohort Study." Am J Clin Nutr **88**(3): 730-737.

Novoselov SV, Calvisi DF, Labunskyy VM, Factor VM, Carlson BA, Fomenko DE, Moustafa ME, Hatfield DL and Gladyshev VN (2005). "Selenoprotein deficiency and high levels of selenium compounds can effectively inhibit hepatocarcinogenesis in transgenic mice." Oncogene **24**(54): 8003-8011.

Nowell PC (1976). "The clonal evolution of tumor cell populations." Science **194**(4260): 23-8.

O'Brien NM, Woods JA, Aherne SA and O'Callaghan YC (2000). "Cytotoxicity, genotoxicity and oxidative reactions in cell-culture models: modulatory effects of phytochemicals." Biochem Soc Trans **28**(2): 22-6.

Oefner PJ, Hunicke-Smith SP, Chiang L, Dietrich F, Mulligan J and Davis RW (1996). "Efficient random subcloning of DNA sheared in a recirculating point- sink flow system." Nucl. Acids Res. **24**(20): 3879-3886.

Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Chan AT, Schernhammer ES, Giovannucci EL and Fuchs CS (2008). "A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer." J Natl Cancer Inst **100**(23): 1734-8.

Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, Suzuki T, Kobayashi A, Yokota J, Sakiyama T, Shibata T, Yamamoto M and Hirohashi S (2008). "Loss of Keap1 Function Activates Nrf2 and Provides Advantages for Lung Cancer Cell Growth." Cancer Research **68**(5): 1303-1309.

Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH and et al. (1994).

"Mutations and altered expression of p16INK4 in human cancer." Proc Natl Acad Sci U S A **91**(23): 11045-9.

Ooi SKT and Bestor TH (2008). "The Colorful History of Active DNA Demethylation." Cell **133**(7): 1145-1148.

Ordway JM, Bedell JA, Citek RW, Nunberg A, Garrido A, Kendall R, Stevens JR, Cao D, Doerge RW, Korshunova Y, Holemon H, McPherson JD, Lakey N, Leon J, Martienssen RA and Jeddeloh JA (2006). "Comprehensive DNA methylation profiling in a human cancer genome identifies novel epigenetic targets." Carcinogenesis **27**(12): 2409-2423.

Paluszczak J, Krajka-Kuzniak V and Baer-Dubowska W (2010). "The effect of dietary polyphenols on the epigenetic regulation of gene expression in MCF7 breast cancer cells." Toxicol Lett **192**(2): 119-25.

Papp LV, Lu J, Holmgren A and Khanna KK (2007). "From Selenium to Selenoproteins: Synthesis, Identity, and Their Role in Human Health." Antioxidants & Redox Signaling **9**(7): 775-806.

Pastore A, Federici G, Bertini E and Piemonte F (2003). "Analysis of glutathione: implication in redox and detoxification." Clinica Chimica Acta **333**(1): 19-39.

Paz MF, Fraga MF, Avila S, Guo M, Pollan M, Herman JG and Esteller M (2003). "A Systematic Profile of DNA Methylation in Human Cancer Cell Lines." Cancer Res **63**(5): 1114-1121.

Peng D, Razvi MH, Chen H, Washington K, Roessner A, Schneider-Stock R and El-Rifai W (2008). "DNA hypermethylation regulates the expression of members of the Mu-class Glutathione-S-Transferases and Glutathione Peroxidases in Barrett's-related adenocarcinomas." Gut.

Petri N, Tannergren C, Holst B, Mellon FA, Bao Y, Plumb GW, Bacon J, O'Leary KA, Kroon PA, Knutson L, Forsell P, Eriksson T, Lennernas H and Williamson G (2003). "Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo." Drug Metabolism and Disposition **31**(6): 805-813.

Pi J, Bai Y, Reece JM, Williams J, Liu D, Freeman ML, Fahl WE, Shugar D, Liu J, Qu W, Collins S and Waalkes MP (2007). "Molecular mechanism of human Nrf2 activation and degradation: Role of sequential phosphorylation by protein kinase CK2." Free Radical Biology and Medicine **42**(12): 1797-1806.

Powis G, Gasdaska JR, Gasdaska PY, Berggren M, Kirkpatrick DL, Engman L, Cotgreave IA, Angulo M and Baker A (1997). "Selenium and the thioredoxin redox system: effects on cell growth and death." Oncol Res **9**(6-7): 303-12.

Prochaska HJ, Santamaria AB and Talalay P (1992). "Rapid detection of inducers of enzymes that protect against carcinogens." Proc Natl Acad Sci U S A **89**(6): 2394-8.

Purdom-Dickinson SE, Sheveleva EV, Sun H and Chen QM (2007). "Translational control of nrf2 protein in activation of antioxidant response by oxidants." Mol Pharmacol **72**(4): 1074-81.

Raffel J, Bhattacharyya AK, Gallegos A, Cui H, Einspahr JG, Alberts DS and Powis G (2003). "Increased expression of thioredoxin-1 in human colorectal cancer is associated with decreased patient survival." J Lab Clin Med **142**(1): 46-51.

Raizis AM, Schmitt F and Jost JP (1995). "A Bisulfite Method of 5-Methylcytosine Mapping That Minimizes Template Degradation." Analytical Biochemistry **226**(1): 161-166.

Ramos-Gomez M, Kwak M-K, Dolan PM, Itoh K, Yamamoto M, Talalay P and Kensler TW (2001). "Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice." Proceedings of the National Academy of Sciences of the United States of America **98**(6): 3410-3415.

Ray K (2010). "New prognostic markers for CRC." Nat Rev Clin Oncol **7**(4): 182-182.

Rayman MP (2005). "Selenium in cancer prevention: a review of the evidence and mechanism of action." Proc Nutr Soc **64**(4): 527-42.

Razin A and Cedar H (1991). "DNA methylation and gene expression." Microbiol. Mol. Biol. Rev. **55**(3): 451-458.

Redman C, Scott JA, Baines AT, Basye JL, Clark LC, Calley C, Roe D, Payne CM and Nelson MA (1998). "Inhibitory effect of selenomethionine on the growth of three selected human tumor cell lines." Cancer Lett **125**(1-2): 103-10.

Reilly C (2006). Selenium in food and health. New Yoork, Springer.

Reiss D, Zhang Y and Mager DL (2007). "Widely variable endogenous retroviral methylation levels in human placenta." Nucleic Acids Res **35**(14): 4743-54.

Richardson B (2007). "Primer: epigenetics of autoimmunity." Nat Clin Pract Rheumatol **3**(9): 521-7.

Robertson KD, Keyomarsi K, Gonzales FA, Velicescu M and Jones PA (2000). "Differential mRNA expression of the human DNA methyltransferases

(DNMTs) 1, 3a and 3b during the G(0)/G(1) to S phase transition in normal and tumor cells." Nucleic Acids Res **28**(10): 2108-13.

Roncucci L, Pedroni M, Vaccina F, Benatti P, Marzona L and De Pol A (2000). "Aberrant crypt foci in colorectal carcinogenesis. Cell and crypt dynamics." Cell Proliferation **33**(1): 1-18.

Ross SA (2003). "Diet and DNA methylation interactions in cancer prevention." Ann N Y Acad Sci **983**: 197-207.

Ross SA, Dwyer J, Umar A, Kagan J, Verma M, Van Bemmel DM and Dunn BK (2008). "Introduction: diet, epigenetic events and cancer prevention." Nutrition Reviews **66**(s1): S1-S6.

Rudolf E, Andelova H and Cervinka M (2009). "Activation of several concurrent proapoptic pathways by sulforaphane in human colon cancer cells SW620." Food Chem Toxicol **47**(9): 2366-73.

Rudolf E, Kralova V and Cervinka M (2008). "Selenium and colon cancer--from chemoprevention to new treatment modality." Anticancer Agents Med Chem **8**(6): 598-602.

Rundlof AK, Carlsten M and Arner ES (2001). "The core promoter of human thioredoxin reductase 1: cloning, transcriptional activity, and Oct-1, Sp1, and Sp3 binding reveal a housekeeping-type promoter for the AU-rich element-regulated gene." J Biol Chem **276**(32): 30542-51.

Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H and Hirohashi S (2003). "Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas." International Journal of Cancer **105**(4): 527-532.

Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K and Ichijo H (1998). "Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1." EMBO J **17**(9): 2596-606.

Schroeder CP, Goeldner EM, Schulze-Forster K, Eickhoff CA, Holtermann P and Heidecke H (2004). "Effect of selenite combined with chemotherapeutic agents on the proliferation of human carcinoma cell lines." Biol Trace Elem Res **99**(1-3): 17-25.

Schröterová L, Králová V, Vorácová A, Hasková P, Rudolf E and Cervinka M (2009). "Antiproliferative effects of selenium compounds in colon cancer cells: Comparison of different cytotoxicity assays." Toxicology in Vitro **23**(7): 1406-1411.

Schulz WA (2006). "L1 retrotransposons in human cancers." J Biomed Biotechnol **2006**(1): 83672.

Schumacher A, Kapranov P, Kaminsky Z, Flanagan J, Assadzadeh A, Yau P, Virtanen C, Winegarden N, Cheng J, Gingeras T and Petronis A (2006). "Microarray-based DNA methylation profiling: technology and applications." Nucleic Acids Res **34**(2): 528-42.

Shamberger RJ and Frost DV (1969). "Possible protective effect of selenium against human cancer." Can Med Assoc J **100**(14): 682.

Shen G, Xu C, Chen C, Hebbar V and Kong AN (2006). "p53-independent G1 cell cycle arrest of human colon carcinoma cells HT-29 by sulforaphane is associated with induction of p21CIP1 and inhibition of expression of cyclin D1." Cancer Chemother Pharmacol **57**(3): 317-27.

Shen L, Ahuja N, Shen Y, Habib NA, Toyota M, Rashid A and Issa J-PJ (2002). "DNA Methylation and Environmental Exposures in Human Hepatocellular Carcinoma." J. Natl. Cancer Inst. **94**(10): 755-761.

Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K, Hamilton SR and Issa J-PJ (2007). "Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer." Proceedings of the National Academy of Sciences **104**(47): 18654-18659.

Shuji O, Takako K, Katsuhiko N, Mutsuko O, Yuko S, Gregory JK and Charles SF (2008). "LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer." International Journal of Cancer **122**(12): 2767-2773.

Si J, Boumber YA, Shu J, Qin T, Ahmed S, He R, Jelinek J and Issa JP (2010). "Chromatin remodeling is required for gene reactivation after decitabine-mediated DNA hypomethylation." Cancer Res **70**(17): 6968-77.

Smart DK, Ortiz KL, Mattson D, Bradbury CM, Bisht KS, Sieck LK, Brechbiel MW and Gius D (2004). "Thioredoxin reductase as a potential molecular target for anticancer agents that induce oxidative stress." Cancer Res **64**(18): 6716-24.

Son H-Y, Nishikawa A, Furukawa F, Lee I-S, Ikeda T, Miyauchi M, Nakamura H and Hirose M (2000). "Modifying effects of 4-phenylbutyl isothiocyanate on N-nitrosobis(2-oxopropyl)amine-induced tumorigenesis in hamsters." Cancer Letters **160**(2): 141-147.

Song J, Sohn KJ, Medline A, Ash C, Gallinger S and Kim YI (2000). "Chemopreventive effects of dietary folate on intestinal polyps in Apc+/-Msh2-/- mice." Cancer Res **60**(12): 3191-9.

Spallholz JE (1994). "On the nature of selenium toxicity and carcinostatic activity." Free Radic Biol Med **17**(1): 45-64.

Squires JE, Stoytchev I, Forry EP and Berry MJ (2007). "SBP2 Binding Affinity Is a Major Determinant in Differential Selenoprotein mRNA Translation and Sensitivity to Nonsense-Mediated Decay." Mol. Cell. Biol. **27**(22): 7848-7855.

Steinmetz KA and Potter JD (1991). "Vegetables, fruit, and cancer. I. Epidemiology." Cancer Causes Control **2**(5): 325-57.

Stone WL, Krishnan K and Papas A (2004). Oxidants and antioxidants in the pathology of colon cancer. In: Redox-genome interactions in health and disease. Fuchs J. J., M. Podda and L. Packer. New York, Marcel Dekker**:** xviii, 629 p.

Stranges S, Laclaustra M, Ji C, Cappuccio FP, Navas-Acien A, Ordovas JM, Rayman M and Guallar E (2009). "Higher selenium status is associated with adverse blood lipid profile in British adults." J Nutr **140**(1): 81-7.

Stranges S, Marshall JR, Natarajan R, Donahue RP, Trevisan M, Combs GF, Cappuccio FP, Ceriello A and Reid ME (2007). "Effects of long-term selenium supplementation on the incidence of type 2 diabetes: a randomized trial." Ann Intern Med **147**(4): 217-23.

Sun L, Zhao H, Xu Z, Liu Q, Liang Y, Wang L, Cai X, Zhang L, Hu L, Wang G and Zha X (2007). "Phosphatidylinositol 3-kinase/protein kinase B pathway stabilizes DNA methyltransferase I protein and maintains DNA methylation." Cellular Signalling **19**(11): 2255-2263.

Szyf M, Bozovic V and Tanigawa G (1991). "Growth regulation of mouse DNA methyltransferase gene expression." J Biol Chem **266**(16): 10027-30.

Szyf M and Detich N (2001). "Regulation of the DNA methylation machinery and its role in cellular transformation." Prog Nucleic Acid Res Mol Biol **69**: 47-79.

Takashi S, Keiji W, Hitoshi N and Minako N (2004). "Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish." Cancer Science **95**(4): 290-299.

Talalay P, Fahey JW, Holtzclaw WD, Prestera T and Zhang Y (1995). "Chemoprotection against cancer by phase 2 enzyme induction." Toxicol Lett **82-83**: 173-9.

Tanaka T, Iwasa Y, Kondo S, Hiai H and Toyokuni S (1999). "High incidence of allelic loss on chromosome 5 and inactivation of p15INK4B and p16INK4A tumor suppressor genes in oxystress-induced renal cell carcinoma of rats." Oncogene **18**(25): 3793-7.

Tang L, Zirpoli GR, Jayaprakash V, Reid ME, McCann SE, Nwogu CE, Zhang Y, Ambrosone CB and Moysich KB (2010). "Cruciferous vegetable

intake is inversely associated with lung cancer risk among smokers: a case-control study." BMC Cancer **10**: 162.

Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M and Biswal S (2002). "Identification of Nrf2-regulated Genes Induced by the Chemopreventive Agent Sulforaphane by Oligonucleotide Microarray." Cancer Research **62**(18): 5196-5203.

Ting AH, Jair KW, Suzuki H, Yen RW, Baylin SB and Schuebel KE (2004). "CpG island hypermethylation is maintained in human colorectal cancer cells after RNAi-mediated depletion of DNMT1." Nat Genet **36**(6): 582-4.

Tominaga K, Fujii S, Mukawa K, Fujita M, Ichikawa K, Tomita S, Imai Y, Kanke K, Ono Y, Terano A, Hiraishi H and Fujimori T (2005). "Prediction of Colorectal Neoplasia by Quantitative Methylation Analysis of Estrogen Receptor Gene in Nonneoplastic Epithelium from Patients with Ulcerative Colitis." Clin Cancer Res **11**(24): 8880-8885.

Tomso DJ and Bell DA (2003). "Sequence context at human single nucleotide polymorphisms: overrepresentation of CpG dinucleotide at polymorphic sites and suppression of variation in CpG islands." J Mol Biol **327**(2): 303-8.

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB and Issa JP (1999). "CpG island methylator phenotype in colorectal cancer." Proc Natl Acad Sci U S A **96**(15): 8681-6.

Traka M, Gasper AV, Smith JA, Hawkey CJ, Bao Y and Mithen RF (2005). "Transcriptome analysis of human colon Caco-2 cells exposed to sulforaphane." J Nutr **135**(8): 1865-72.

Trujillo E, Davis C and Milner J (2006). "Nutrigenomics, proteomics, metabolomics, and the practice of dietetics." J Am Diet Assoc **106**(3): 403-13.

Uden PC, Boakye HT, Kahakachchi C, Hafezi R, Nolibos P, Block E, Johnson S and Tyson JF (2004). "Element selective characterization of stability and reactivity of selenium species in selenized yeast." Journal of Analytical Atomic Spectrometry **19**(1): 65-73.

Ueno M, Masutani H, Arai RJ, Yamauchi A, Hirota K, Sakai T, Inamoto T, Yamaoka Y, Yodoi J and Nikaido T (1999). "Thioredoxin-dependent Redox Regulation of p53-mediated p21 Activation." Journal of Biological Chemistry **274**(50): 35809-35815.

Valko M, Rhodes CJ, Moncol J, Izakovic M and Mazur M (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." Chemico-Biological Interactions **160**(1): 1-40.

Van Steensel B (2005). "Mapping of genetic and epigenetic regulatory networks using microarrays." Nat Genet.

Verhoeven DT, Goldbohm RA, van Poppel G, Verhagen H and van den Brandt PA (1996). "Epidemiological studies on brassica vegetables and cancer risk." Cancer Epidemiol Biomarkers Prev **5**(9): 733-748.

Visanji JM, Duthie SJ, Pirie L, Thompson DG and Padfield PJ (2004). "Dietary Isothiocyanates Inhibit Caco-2 Cell Proliferation and Induce G2/M Phase Cell Cycle Arrest, DNA Damage, and G2/M Checkpoint Activation." J. Nutr. **134**(11): 3121-3126.

Wang LG, Beklemisheva A, Liu XM, Ferrari AC, Feng J and Chiao JW (2007). "Dual action on promoter demethylation and chromatin by an isothiocyanate restored GSTP1 silenced in prostate cancer." Molecular Carcinogenesis **46**(1): 24-31.

Wang W, Wang S, Howie AF, Beckett GJ, Mithen R and Bao Y (2005). "Sulforaphane, Erucin, and Iberin Up-Regulate Thioredoxin Reductase 1 Expression in Human MCF-7 Cells." J. Agric. Food Chem. **53**(5): 1417-1421.

Wang X-J, Sun Z, Villeneuve NF, Zhang S, Zhao F, Li Y, Chen W, Yi X, Zheng W, Wondrak GT, Wong PK and Zhang DD (2008). "Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2." Carcinogenesis **29**(6): 1235-1243.

Warin R, Chambers WH, Potter DM and Singh SV (2009). "Prevention of Mammary Carcinogenesis in MMTV-neu Mice by Cruciferous Vegetable Constituent Benzyl Isothiocyanate." Cancer Research **69**(24): 9473-9480.

Wasson GR, McGlynn AP, McNulty H, O'Reilly SL, McKelvey-Martin VJ, McKerr G, Strain JJ, Scott J and Downes CS (2006). "Global DNA and p53 Region-Specific Hypomethylation in Human Colonic Cells Is Induced by Folate Depletion and Reversed by Folate Supplementation." J. Nutr. **136**(11): 2748-2753.

WCRF/AICR (1997). Food, nutrition and the prevention of cancer : a global perspective. Washington, D.C., American Institute for Cancer Research.

WCRF/AICR (2007). Food, nutrition, physical activity, and the prevention of cancer [electronic resource] : a global perspective. Washington, D.C., American Institute for Cancer Research.

Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL and Schubeler D (2005). "Chromosome-wide and promoter-specific analyses identify

sites of differential DNA methylation in normal and transformed human cells." Nat Genet **37**(8): 853-62.

Wei WQ, Abnet CC, Qiao YL, Dawsey SM, Dong ZW, Sun XD, Fan JH, Gunter EW, Taylor PR and Mark SD (2004). "Prospective study of serum selenium concentrations and esophageal and gastric cardia cancer, heart disease, stroke, and total death." Am J Clin Nutr **79**(1): 80-5.

Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R and Laird PW (2006). "CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer." Nat Genet **38**(7): 787-793.

Willett WC (2010). "Fruits, Vegetables, and Cancer Prevention: Turmoil in the Produce Section." J. Natl. Cancer Inst. **102**(8): 510-511.

Wingler K, Bocher M, Flohe L, Kollmus H and Brigelius-Flohe R (1999). "mRNA stability and selenocysteine insertion sequence efficiency rank gastrointestinal glutathione peroxidase high in the hierarchy of selenoproteins." Eur J Biochem **259**(1-2): 149-57.

Wingler K and Brigelius-Flohe R (1999). "Gastrointestinal glutathione peroxidase." Biofactors **10**(2/3): 245.

Wong ML and Medrano JF (2005). "Real-time PCR for mRNA quantitation." Biotechniques **39**(1): 75-85.

Xiang N, Zhao R, Song G and Zhong W (2008). "Selenite reactivates silenced genes by modifying DNA methylation and histones in prostate cancer cells." Carcinogenesis **29**(11): 2175-2181.

Xiang N, Zhao R and Zhong W (2009). "Sodium selenite induces apoptosis by generation of superoxide via the mitochondrial-dependent pathway in human prostate cancer cells." Cancer Chemother Pharmacol **63**(2): 351-62.

Xiong Z and Laird PW (1997). "COBRA: a sensitive and quantitative DNA methylation assay." Nucl. Acids Res. **25**(12): 2532-2534.

Xu XL, Yu J, Zhang HY, Sun MH, Gu J, Du X, Shi DR, Wang P, Yang ZH and Zhu JD (2004). "Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis." World J Gastroenterol **10**(23): 3441-54.

Yamaji T, Inoue M, Sasazuki S, Iwasaki M, Kurahashi N, Shimazu T and Tsugane S (2008). "Fruit and vegetable consumption and squamous cell

carcinoma of the esophagus in Japan: The JPHC study." International Journal of Cancer **123**(8): 1935-1940.

Yamamoto Y and Takahashi K (1993). "Glutathione peroxidase isolated from plasma reduces phospholipid hydroperoxides." Arch Biochem Biophys **305**(2): 541-5.

Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH and Issa JP (2004). "A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements." Nucleic Acids Res **32**(3): e38.

Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA and Talalay P (2002). "Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans." Clinica Chimica Acta **316**(1-2): 43-53.

Yoo M-H, Xu X-M, Carlson BA, Patterson AD, Gladyshev VN and Hatfield DL (2007). "Targeting Thioredoxin Reductase 1 Reduction in Cancer Cells Inhibits Self-Sufficient Growth and DNA Replication." PLoS ONE **2**(10): e1112.

Yoon SO, Kim MM and Chung AS (2001). "Inhibitory effect of selenite on invasion of HT1080 tumor cells." J Biol Chem **276**(23): 20085-92.

Yoon SO, Kim MM, Park SJ, Kim D, Chung J and Chung AS (2002). "Selenite suppresses hydrogen peroxide-induced cell apoptosis through inhibition of ASK1/JNK and activation of PI3-K/Akt pathways." FASEB J **16**(1): 111-3.

Yoshiyuki M, Hiroyuki T, Takamasa K, Kenichi H, Kazuo Y, Kimiyasu N, Osamu T, Michimori K, Tomoyuki M, Eiji N, Haruhiko K, Tatsuya M, Kazuhiko T, Mitsuhiko O, Hisao I, Junji Y, Yoshikazu M and Goshi S (2008). "Aberrant expression of selenoproteins in the progression of colorectal cancer." Cancer Letters **259**(2): 218-230.

Yu S, Khor TO, Cheung KL, Li W, Wu TY, Huang Y, Foster BA, Kan YW and Kong AN (2010). "Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice." PLoS One **5**(1): e8579.

Yuasa Y, Nagasaki H, Akiyama Y, Sakai H, Nakajima T, Ohkura Y, Takizawa T, Koike M, Tani M, Iwai T, Sugihara K, Imai K and Nakachi K (2005). "Relationship between CDX2 gene methylation and dietary factors in gastric cancer patients." Carcinogenesis **26**(1): 193-200.

Zeng H, Botnen JH and Johnson LK (2008). "A selenium-deficient Caco-2 cell model for assessing differential incorporation of chemical or food

selenium into glutathione peroxidase." Biol Trace Elem Res **123**(1-3): 98-108.

Zeng H and Combs Jr GF (2008). "Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion." The Journal of Nutritional Biochemistry **19**(1): 1-7.

Zhang J, Svehlikova V, Bao Y, Howie AF, Beckett GJ and Williamson G (2003). "Synergy between sulforaphane and selenium in the induction of thioredoxin reductase 1 requires both transcriptional and translational modulation." Carcinogenesis **24**(3): 497-503.

Zhang Y (2004). "Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **555**(1-2): 173-190.

Zhang Y and Callaway EC (2002). "High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate." Biochem J **364**(Pt 1): 301-7.

Zhang Y and Talalay P (1994). "Anticarcinogenic Activities of Organic Isothiocyanates: Chemistry and Mechanisms." Cancer Res **54**(7_Supplement): 1976s-1981.

Zhang Y and Talalay P (1998). "Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes." Cancer Res **58**(20): 4632-9.

Zhang Y, Talalay P, Cho CG and Posner GH (1992). "A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure." Proceedings of the National Academy of Sciences of the United States of America **89**(6): 2399-2403.

Zhang Y, Tang L and Gonzalez V (2003). "Selected isothiocyanates rapidly induce growth inhibition of cancer cells." Mol Cancer Ther **2**(10): 1045-1052.

Zhang Y, Yao S and Li J (2006). "Vegetable-derived isothiocyanates: anti-proliferative activity and mechanism of action." Proc Nutr Soc **65**(1): 68-75.

Zhang ZM, Yang XY, Yuan JH, Sun ZY and Li YQ (2009). "Modulation of NRF2 and UGT1A expression by epigallocatechin-3-gallate in colon cancer cells and BALB/c mice." Chin Med J (Engl) **122**(14): 1660-5.

Zhao R and Holmgren A (2002). "A Novel Antioxidant Mechanism of Ebselen Involving Ebselen Diselenide, a Substrate of Mammalian Thioredoxin and Thioredoxin Reductase." Journal of Biological Chemistry **277**(42): 39456-39462.

Zhu J-K (2009). "Active DNA Demethylation Mediated by DNA Glycosylases." Annual Review of Genetics **43**(1): 143-166.

Zhuo H, Smith AH and Steinmaus C (2004). "Selenium and lung cancer: a quantitative analysis of heterogeneity in the current epidemiological literature." Cancer Epidemiol Biomarkers Prev **13**(5): 771-8.