Cell cycle effects of L-sulforaphane, a major antioxidant from cruciferous vegetables: The role of the anaphase promoting complex

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Abstract

L-sulforaphane (LSF) is a natural isothiocyanate found in cruciferous vegetables particularly broccoli. LSF has been identified as a potent antioxidant and anti-cancer agent and is widely known to regulate phase II detoxifying enzymes and induce cell cycle arrest or apoptosis in malignant cells *in vitro* and *in vivo*. Previous studies have found significant G2/M cell cycle arrest in response to LSF in various model of cancer and results have mainly been attributed to increased cyclin B1 protein levels and increased p21expression. Using genome-wide mRNA-Seq analysis we provide insights into the molecular mechanisms of action of LSF to identify a key pathway in cell cycle progression - the role of the anaphase promoting complex (APC) pathway. We evaluated gene expression changes in human erythroleukemic K562 cells following treatment with 15μM LSF for 48h and compared them to immortalized human keratinocytes, human microvascular endothelial cells (HMEC-1) cells and normal human umbilical endothelial cells (HUVEC). *We identified* disparate gene expression changes in response to LSF between malignant and normal cells and immortalized cell lines. *The results highlight* significant down-regulation of kinase CDK1 which is suggestive that the existence and activity of APC/CDC20 complex will be inhibited along with its associated down-stream degradation of key cell cycle regulators preventing cell cycle progression from mitotic exit.

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Introduction

It has been demonstrated that the consumption of cruciferous vegetables such as cauliflower, watercress, brussel sprouts, and broccoli, is associated with reduced risks of cancer at various sites, particularly colon, lung and prostate [1]. This protective effect is attributed to isothiocyanates, one in particular named L-sulforaphane (LSF) [2, 3]. Cruciferous vegetables have high glucosinolate content. Hydrolysis of glucoraphanin (a glucosinolate) into LSF can be catalysed by myrosinase enzymes which are released by the vegetables as well as being present in the gastrointestinal tract [1, 4, 5]. The metabolism of LSF results in the generation of sulforaphane-cysteine (SFN-cys), followed by sulforaphane-N-acetylcysteine (SFN-NAC) [6] and its these downstream products which have identified LSF as a as potent antioxidant and natural inducer of phase 2 detoxification enzymes [1]. Since its discovery, numerous research has investigated the efficacy of LSF to induce phase 2 enzymes and subsequently protect cells from reactive oxygen species and oxidative stress [7, 8].

Furthermore, LSF has also been known to inhibit HDAC activity by direct binding to HDAC enzymes. Aberrant expressions of various HDAC enzymes have been implicated in various cancers and are known to suppress tumour suppressor genes [9]. Since HDACs are commonly dysregulated in transformed cells, inhibiting HDAC activity has been considered an important therapeutic intervention for cancer. HDAC inhibitors can discriminately alter gene expression by acetylating histones as well as proteins involved in regulating transcription [10]. In doing so, they are able to induce cell cycle arrest and cell death preferentially in malignant cell lines [11]. Cell cycle arrest, mediated by HDAC inhibitors, occurs as a result of acetylation of the histone associated with the promotor region of CKIs (cyclin-dependent kinase inhibitors). Previous findings have found CKI and p21 is predominantly affected by HDAC inhibitors. Its expression is increased and it is able to act on CDKs, and thus inhibit cell growth [10, 12, 13]. HDAC inhibitors are able to induce cell cycle arrest in both proliferating and non-proliferating cells. This sets them apart from chemotherapy drugs, which are only successful in inhibiting cell growth in non-proliferating cells [10]. LSF's ability to target aberrant epigenetic patterns makes it an effective chemopreventative at multiple stages of the tumorigenesis pathway [1]. Previous findings have identified various cellular pathways to be altered in response to LSF treatment in malignant cell lines including; induction of apoptotic pathways, suppression of cell cycle progression and anti-inflammatory activities [1, 5, 6, 14-16]. The anti-cancer properties of LSF has been broadly attributed to its HDAC inhibitory mechanism – it is suggested that global acetylation of histone and non-histone proteins results in the overexpression of pro-apoptotic proteins and cyclins, inducing cell cycle arrest and apoptosis via multicaspase activation, thereby hindering tumour progression [6, 14]. Despite its known biological affects in malignant cells, the molecular mechanism of LSF is still not fully elucidated.

In the present study we aim to provide insights into the molecular mechanisms of action of LSF using genome-wide mRNA-Seq analysis. In particular we interrogate the role of the anaphase promoting complex (APC) in cell cycle

progression pathway and evaluated gene expression changes in human erythroleukemic K562 cells following treatment with 15μM LSF. In addition, we compare LSF induced-gene expression changes found in the malignant K562 cells to transformed human keratinocytes and human microvascular endothelial cells (HMEC-1) cells and the normal human umbilical endothelial cells (HUVEC) to identify the efficacy of LSF as a anti-cancer modality as well to further understand its mechanism of action to induce cell cycle arrest preferentially in malignant cell lines.

Materials and methods

Cell culture

Human chronic myelogenous leukaemia K562 cells [1], were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in complete-Royal Park Memorial Institute (RPMI) 1640 medium supplemented with 20mmol/L HEPES (pH 7.4; GIBCO-Invitrogen, Carlsbad, CA, USA), 10% (v/v) fetal bovine serum (FBS; In vitro Technologies, VIC, AUS), 2mM/L L-glutamine (GIBCO-Invitrogen), and 20μg/mL gentamicin (GIBCO-Invitrogen). Cells were cultured in suspension in a humidified atmosphere of 5% (v/v) CO_2 at 37°C and were maintained in exponential growth phase. For maintenance, cells were passaged twice per week and seeded at ratios 1:10.

Human neonatal foreskin keratinocytes transfected with the human papilloma virus (HPV), kindly provided by Dr. Pritinder Kaur (Peter McCallum Cancer Institute) were grown as monolayers in keratinocyte-Serum Free Medium (SFM) medium (GIBCO-Invitrogen) supplemented with L-glutamine, human epidermal growth factor (EGF), and bovine pituitary extract (BPE). Human microvascular endothelial cells (HMEC-1) transfected with the rous sarcoma virus were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown as monolayers in MCDB-131 medium (GIBCO-Invitrogen) supplemented with L-glutamine, epidermal growth factor (EGF), heparin, hydrocortisone and Gluta-MAX (GIBCO-Invitrogen). Human umbilical vein endothelial cells (HUVECs, Technoclone, Vienna, Austria) were grown in monolayers in endothelial cell growth medium (ECM-2) supplemented with 100U/mL penicillin, 100μg/mL streptomycin, 0.25μg/mL fungizone, 2mM L-glutamine, 5U/mL heparin, 30-50μg/mL endothelial cell growth supplement (Technoclone, Vienna, Austria). All cells were maintained in the exponential growth phase in T75cm^2 vented culture flasks and passaged by brief trypsination (0.05% (v/v) trypsin-EDTA; GIBCO, Invitrogen) before seeding at ratios of 1:3; 37°C, 5% (v/v) CO_2.

Cell treatments

All cell lines were seeded at cell densities of 5x10^5 cells/mL in T75cm^2 vented culture flasks. For adherent cell lines, cells were allowed to attach overnight before treatment with 15μM L-sulforaphane (Sigma-Aldrich) for 48h at 37°C, 5% (v/v) CO_2.

RNA isolation, mRNA sequencing and pathway analysis

Total RNA was isolated from the cells using Trizol (Invitrogen) preparation following manufacturer's protocol. RNA integrity and concentration was measured with the RNA kit and MultiNA capillary electrophoresis system (Shimadzu). Library preparation, alignment to the genome and bioinformatics analysis to obtain fold changes in mRNA expression was performed as previously described [29]. The online program MetaCore (GeneGo Inc. St. Joseph, MI, USA) was used for pathway analysis.

Flow cytometric analysis

Flow cytometry was utilized for cell cycle analysis. K562 cells were treated 15μM LSF for 48h before brief centrifugation to pellet the cells. After removal of the medium, cells were washed with ice-cold PBS containing 2% (v/v) FBS twice and fixed overnight with continuous rotation in 70% (v/v) ethanol at 4°C. Cells where then stained in 100μg/mL propidium iodide containing 1μg/mL RNase A (Qiagen Inc., Valencia, CA), overnight whilst on rotation at 4°C in the dark. Samples were then transferred to FACS tubes and analysed by flow cytometry using a FACS Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescent debris was gated using forward scatter versus orthogonal side scatter with a secondary gate placed around the single cell population using a pulse area versus plate width dot plot. The percentage of cells in each phase of the cell cycle was analysed using Cell Quest software (version 5.1, BD Biosciences, San Jose, CA, USA).

Results

Effects of LSF on gene expression in the APC cell cycle regulation pathway. We utilised mRNA sequencing performed by next-generation sequencing (NGS) at a depth of approximately 20 million mapped reads per samples to determine the effect of LSF on gene expression in K562, keratinocytes, HMEC-1 and HUVEC cells treated with 15μM LSF for 48h. Reads were generated by the Illumina base calling software V1.6 and aligned using Burrows Wheeler Aligner to the human transcriptome database curated by RefSeq (Hg18 build). Raw counts were normalised to score reads per transcript per million reads. These scores were used to determine relative fold changes, with significant changes in expression defined by ±1.5-fold change. The online program MetaCore (GeneGo Inc., St. Joseph, MI, USA) was used for pathway analysis. Initially analysis of the top 10 significant pathways affected by LSF treatment across all four cell lines was performed to find the role of APC in cell cycle regulation pathways highly significant. Given that the APC pathway is a crucial pathway for cell division progression we interrogated gene expression changes related to protein degradation in an APC-dependent manner (Table 1). We found 90% of the genes involved in this pathway were either up- and down-

regulated in response to LSF in either of the four cell lines examined and the K562 cell line had the most gene differentially expressed in response to LSF. Genes which had a fold change greater than 10 included: CCT3 (-12.53 fold), CDC16 (-17.9 fold) and FBX05 (-17.01 f old) in K562s and CCNB2 (-16.83 fold), CDC20 (-26.82 fold) and PLK1 (-12.63 fold) in HUVECs. In addition, we found that genes were mainly down-regulated by LSF treatment across the four cell lines and genes were only found to be up-regulated in the K562 and HMEC-1 cell lines (Fig. 1).

Table 1. *Gene expression changes of genes involved in cell cycle regulation mediated by the anaphase promoting complex (APC) following 48h treatment with L-sulforaphane in malignant erythroleukemic K562 cell, transformed keratinocytes, transformed human microvascular endothelial cells (HMEC-1) and human umbilical vein endothelial cells (HUVEC)*

Gene Name	Gene description	Fold change			
		K562	HMEC-1	K'cytes	HUVEC
ANAPC1	Anaphase promoting complex subunit 1	-1.65			
ANAPC2	Anaphase promoting complex subunit 2	-1.59			
ANAPC10	Anaphase promoting complex subunit 10	1.56			
ANAPC11	Anaphase promoting complex subunit 11	1.94	2.23		
AURKA	Serine/threonine protein kinase 6 (aurora kinase A)	2.70		-1.61	-1.74
BUB1	Mitotic checkpoint serine/threonine-protein kinase BUB1	1.71			
BUB3	Mitotic checkpoint protein BUB3	-1.65	-3.56		
CCNA2	Cyclin A2	1.80		-1.58	-8.98
CCNB1	G2/mitotic-specific cyclin-B1	2.40		-1.70	-9.97
CCNB2	G2/mitotic-specific cyclin-B2	2.66		-1.86	-16.83
CCT3	Chaperonin containing TCP1, subunit 3 (gamma)	-12.53	-7.86		
CCT6A	Chaperonin containing TCP1, subunit 6A (zeta 1)	-1.56			
CDC6	Cell division control protein 6 homolog				-4.80
CDC16	Cell division cycle protein 16 homolog	-17.90			
CDC20	Cell division cycle protein 20 homolog	2.37		-1.64	-26.82
CDC23	Cell division cycle protein 23 homolog		1.84		
CDC25A	M-phase inducer phosphatase 1		1.52		
CDC27	Cell division cycle protein 27 homolog		-2.84		
CDCA3	Cell division cycle-associated protein 3 (Tome-1)			-1.60	
CDK1	Cyclin-dependent kinase 1	-2.05	2.56	-1.79	
CDK2	Cyclin-dependent kinase 2				-1.62
CDK2AP1	Cyclin-dependent kinase 2 associated protein 1	1.53		-1.74	
CDK2AP2	Cyclin-dependent kinase 2 associated protein 2			-1.97	-1.68
CKS1B	Cyclin-dependent kinases regulatory subunit 1	1.75		-1.56	-3.65
FBXO5	F-box only protein 5 (Emi1)	-17.01	-8.00		
FZR1	Fizzy-related protein homolog (hCDH1)	-1.82	-1.72	-1.72	
GMNN	Geminin, DNA replication inhibitor				-2.90
KIF22	Kinesin-like protein KIF22 (Kid)	1.60			-2.91
NEK2	Serine/threonine-protein kinase Nek2	2.54		-1.64	
PLK1	Polo-like kinase 1		-1.52	-1.66	-12.63
PTTG1	Securin	2.02		-1.52	-6.40
RASSF1	Ras association domain-containing protein 1	1.84	1.59		-1.52
SKP2	S-phase kinase-associated protein 2 (p45)	-1.67	-1.77	-1.60	

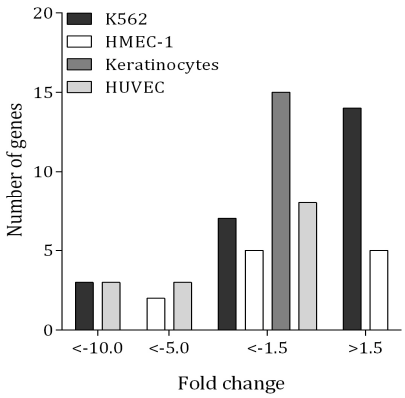

Figure 1. Histograms depicting the number of genes differentially expressed in the anaphase promoting complex (APC) cell cycle regulation pathway following treatment with 15μM L-sulforaphane (LSF). The majority of genes involved in the anaphase promoting complex (APC) cell cycle regulation pathway are down-regulated in response to LSF treatment for 48h in malignant erythroleukemic cells (K562), transformed cells lines; human keratinocytes and human microvascular endothelial cells (HMEC) and normal human umbilical vein endothelial cells (HUVEC).

Secondly we found LSF modifies genes expression in a cell type specific manner across the normal, malignant and transformed cells. Venn diagrams demonstrating the comparison of significant up- or down-regulated gene expression between the four cell lines showed no overlap in genes expression changes between malignant erythroleukemic cells

(K562) when compared to the normal human umbilical vein endothelial cells (HUVEC) (Fig. 2). Overlap of genes occurs between transformed cells lines; human keratinocytes and human microvascular endothelial cells (HMEC) when compared to either malignant K562 cells or normal HUVEC cells. Components in the APC pathway regulate cell cycle progression mainly during G2 to M phase. Cell cycle analysis performed in K562 cells indicates pre-treatment with 15μM LSF for 48h results in significant G2/M cell cycle arrest (Fig. 3).

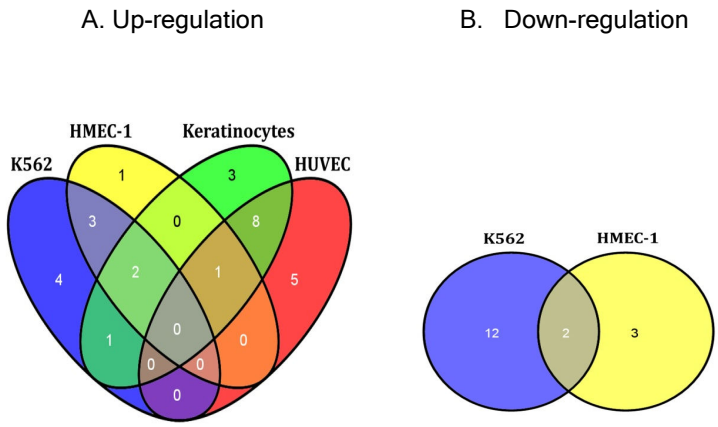

Figure 2. L-sulforaphane (LSF) modifies genes expression in a cell type specific manner in normal, malignant and transformed cells. Venn diagrams demonstrating the overlap in either up-regulated (A) and down-regulated (B) genes following treatment with 15μM LSF for 48h shows cell type specificity in normal, malignant and transformed cell lines with genes involved in the anaphase promoting complex (APC) cell cycle regulation pathway. No overlap in genes expression changes was observed in malignant erythroleukemic cells (K562) when compared to the normal human umbilical vein endothelial cells (HUVEC). Overlap of genes occurs between transformed cells lines; human keratinocytes and human microvascular endothelial cells (HMEC) when compared to either malignant K562 cells or normal HUVEC cells. Genes involved in the APC cell cycle regulation pathway were not down-regulated by LSF in keratinocytes or HUVECs.

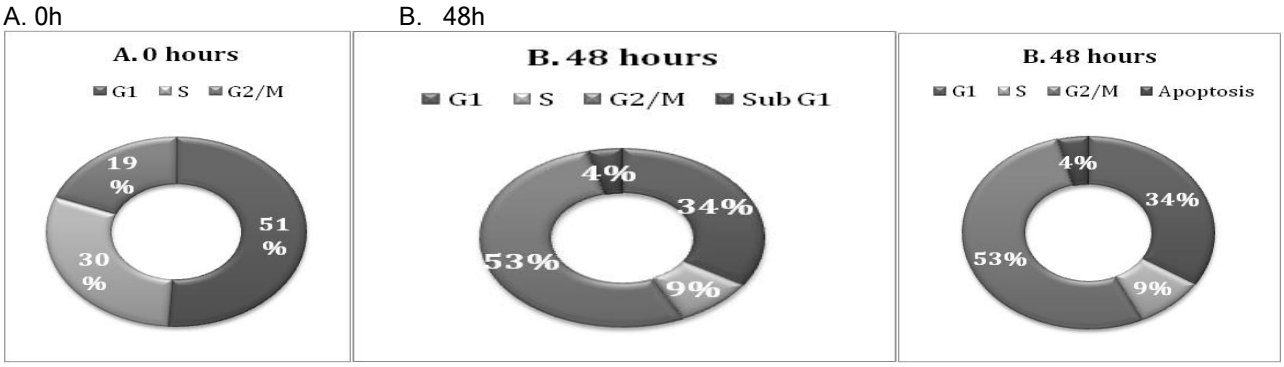

Figure 3. L-sulforaphane causes G2/M cell cycle arrest in human erythroleukemic K562 cells. Flow cytometric analyses of cells were treated with 15μM LSF for 48h prior to staining with propidium iodide (PI). Shown is the percentage of cells in each phase of the cell cycle was analyzed using FlowJo V7.6 flow cytometry analysis.

Discussion

In recent years, the isothiocyanate LSF, found naturally in cruciferous vegetables has been shown to possess anti-cancer and antioxidant effects in a numerous *in vitro* and *in vivo* model systems [17-19]. Biological effects in *in vitro* investigations have found LSF to regulate phase II detoxifying enzymes, cell cycle progression and apoptosis [18, 20-23]. Previous studies performed in HT-29 colorectal cancer cells and acute lymphoblastic leukemia (ALL) cells, have attributed the G2/M cell cycle arrest to increased cyclin B1 protein levels, induced p21 expression and have speculated Cdc2 kinase as a key target for LSF [17, 20]. Although mechanisms of action have been associated in part with histone acetylation via HDAC inhibition and induction of phase II enzymes, little is still known of the molecular mechanism of action of LSF in the concomitant regulation of cell growth in cancer cells.

With decreasing costs, genome-wide differential gene expression studies using microarray or mRNA-Sequencing technologies are increasingly being utilized by individual laboratories. Methodologies for determining statistically significant changes in gene expression between test groups are now well-established, yielding reliable data. Following

quality control and statistical analyses, biological meaning is deciphered by detailed pathway interrogation. Numerous commercial software programs as well as freely available online databases are available for researchers to analyze the functional significance of relevant genes. Typically, on the basis of the differential expression of genes, the software deduces the most highly regulated pathways. Researchers then select the most relevant biological pathway for further scrutiny. This process yields useful information and may allow for identification of novel pathways and new targets for further exploration. However, such analysis largely ignores the complex biological regulation of cellular proteins. Although intuitive, this is increasingly overlooked when describing the findings from genome-wide differential gene expression studies. Here we describe the biology of the anaphase promoting complex (APC), a key regulator of the cell cycle in response to LSF in malignant, transformed and normal cells. Evaluation of the biological regulation of the anaphase promoting complex, highlights the inherent complexities and gives insights into the preferentially cell cycle effects in malignant cells.

APC is a multisubunit E3 ubiquitin ligase which displays is effects by assembling polyubiquitin chains on substrate proteins and targets these proteins for destruction by the 26S proteasome. Cell cycle regulation via the APC pathway occurs through and exits the mitotic phase of the cell cycle and controls entry into the S phase and its unidirectional progression is accomplished by the degradation of key cell-cycle regulators. Division cycle protein 20 homolog (CDC20) is required for activation and substrate recognition of APC mainly during prophase to anaphase [24]. Although CDC20 is upregulated 2.37-fold in K562 cells and down-regulated 26.82-fold in HUVEC cells in response to LSF, phosporylation of APC by cyclin dependent kinase 1 (CDK1) is required for CDC20 to bind to APC and subsequently modulate APC activity (Table 1) [24]. Furthermore, it is proposed that direct phosphorylation of CDC20 by CDK1 or indirect phosphorylation of CDC20 by polo-like kinase 1 (PLK1) is required for CDC20 -dependent APC activation [25] or for APC regulation of the spindle checkpoint [24]. In response to LSF treatment the kinase CDK1 is down-regulated in K562 cells and keratinocytes by 2.05-fold and 1.79-fold respectively, preventing the existence and activity of APC/CDC20 complex. At the spindle checkpoint, mitotic spindle assembly checkpoint protein - budding uninhibited by benzimidazoles 1 homologues (BUB1), may inhibit the APC/CDC20 complex [26]. BUB1 was found to be up-regulated in K562 cells 1.71-fold following LSF treatment. An additional negative regulator of the APC/CDC20 complex, Ras association domain-containing protein 1 (RASSF1) [27] was up-regulated by LSF in K562 and HMEC-1 cells, 1.87 and 1.59-fold respectively (Table 1).

Fizzy-related protein homolog (hCDH1) is responsible for substrate recognition and activation of APC during mitotic exit and G1 respectively [24]. Although the main APC substrate during the G1 phase is the APC activator CDC20, proteolysis of CDC20 by the APC/ hCDH1 complex further induces APC/CDC20 inactivation and allows a switch from APC/CDC20 to APC/ hCDH1 complex [28]. In malignant K562 cells and immortalised keratinocytes and HMEC-1 cells hCDH1 gene expression was down-regulated 1.82, 1.72 and 1.72-fold respectively in response to LSF treatment preventing activation of APC and substrate binding for mitotic exit and cell cycle progression in G1. Finally, cell cycle analysis performed in K562 cells pre-treated with 15μM LSF for 48 hours showed G2/M cell cycle arrest and the findings are analogous with those from the mRNA-Seq analysis (Fig. 3).

Overall, our findings investigating the role of APC in cell cycle progression indicate a molecular mechanism of action of the isothiocyanate, LSF induced G2/M cell cycle arrest in the erythroleukemic K562 cells. Furthermore, highlighted is the discriminatory actions of LSF in regulating gene expression in malignant cells compared the normal HUVEC cell line. Here we show disparate gene expression changes in response to LSF between malignant and normal cells and immortalized cell lines HMEC-1 and keratinocytes. The results highlight significant down-regulation of kinase CDK1 in K562 cells preventing the existence and activity of APC/CDC20 complex and its associated down-stream degradation of key cell cycle regulators inhibiting cell cycle progression from mitotic exit.

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The authors declare that they have no conflicts of interest.

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