Quantitative and Temporal Proteome Analysis of Butyrate-treated Colorectal Cancer Cells

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Summary

Colorectal cancer is one of the most common cancers in developed countries, and its incidence is negatively associated with high dietary fiber intake. Butyrate, a short-chain fatty acid fermentation by-product of fiber induces cell maturation with the promotion of growth arrest, differentiation, and/or apoptosis of cancer cells. The stimulation of cell maturation by butyrate in colonic cancer cells follows a temporal progression, from the early phase of growth arrest to the activation of apoptotic cascades. Previously, we performed 2-D DIGE to identify differentially expressed proteins induced by 24h butyrate treatment of HCT-116 colorectal cancer cells. Herein, we employed quantitative proteomics approaches using iTRAQ, a stable isotope labeling methodology that enables multiplexing of 4 samples, for a temporal study of HCT-116 cells treated with butyrate. In addition, cICAT which selectively tags cysteine-containing proteins was also used, and the results complemented that obtained from the iTRAQ strategy. Selected protein targets were validated by real-time PCR and western blotting. A model is proposed to illustrate our findings from this temporal analysis of butyrate-responsive proteome which uncovered several integrated cellular processes and pathways involved in growth arrest, apoptosis, and metastasis. These signature clusters of butyrate-regulated pathways are potential targets for novel chemopreventive and therapeutic drugs for treatment of colorectal cancer.
Introduction

In developed countries, colorectal cancer is a prevalent disease with high mortality and morbidity rates (1). This disease has emerged as the top malignancy in Singapore. Environmental factors are responsible for about 80% of the cases whereas genetic predisposition accounts for the minority 20% of cases. Epidemiological evidence suggests that high intake of dietary fiber reduces the incidence and risk of this neoplasm (2, 3). A wealth of studies has shown that butyrate produced from anaerobic fermentation of indigestible carbohydrate, is the molecule responsible for the chemopreventive properties of fiber-rich diet (4–6).

While butyrate serves as an energy source for normal colonocytes, in vivo and in vitro studies have shown that at physiological concentrations, this natural short-chain fatty acid mediates cell maturation with the promotion of growth arrest followed by differentiation, and/or apoptosis of cancer cells (7–11). These biological effects are crucial in colorectal cancer therapy as colonic transformation is characterized by multi-stage alterations of tissue homeostasis resulting in aberrant cell division and/or cell death (12, 13). Butyrate has been purported as a potential anti-cancer agent. This initiated notable research in identifying proteins that contribute to its biological effects (14, 15). However, most of these investigations focused on one target at any one time and were thus unable to systematically elucidate butyrate’s mode of actions in an integrated manner.

Through the use of DNA microarray technology, Mariadason et al. (16) showed that butyrate induced maximal genetic reprogramming after 16h of treatment on colorectal cancer cells. In our earlier work, a functional proteomics approach using pre-fractionation strategy coupled with 2-D DIGE analysis was undertaken to identify candidate proteins regulated by 24h butyrate treatment in HCT-116 cells (17). We have also demonstrated the cell line’s high
sensitivity to butyrate-induced growth inhibition and apoptosis in a time- and dose-dependent manner (18). Therefore, the stimulation of cell maturation by butyrate implicated a temporal orchestration of various cellular processes.

In this article, we carried out comparative proteome analysis of HCT-116 cells treated with butyrate at three time-points with the aim to identify clusters of proteins (and pathways) that showed a consistent trend of differential expression over time. The synergistic influence of each cluster of proteins may result in the overall phenotypic response to butyrate. Herein, the chosen period of treatment (24h, 36h and 48h) spans from the induction of growth arrest and early phase of apoptosis till the late phase of cell death. In addition to providing insights into the mechanism underlying butyrate’s pleiotropic effects, our study of the time dynamics of butyrate treatment could lead to the discovery of potential therapeutic targets associated with the progression of cell maturation in cancer cells. As the iTRAQ methodology permits multiplexing of 4 samples in a single experiment, it is well suited for the evaluation of the dynamic cellular response to butyrate in a time-course study (19). Here, we show the first experimental iTRAQ data for butyrate-treated HCT-116 cells carried out at 24, 36 and 48 hours.

**Experimental Procedures**

**Cell culture.** HCT-116 colorectal cancer cells were cultured and treated with 5mM sodium butyrate as previously reported, except that 3 treatment time-points (24h, 36h, and 48h) were used (17).

**iTRAQ labeling.** Four batches each of control cells (24h mock-treated), and cells treated with 5mM sodium butyrate for 24h, 36h, and 48h respectively were harvested. 500mM
triethylammonium bicarbonate/1.0% (w/v) SDS was used for extraction and denaturation of cellular proteins by boiling at 100°C for 10min. Cellular debris was removed after centrifugation at 18,800g for 1h at 23°C. iTRAQ labeling of each sample was performed according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). 100µg of protein was reduced with 5mM tris-(2-carboxyethyl)phosphine (TCEP) at 60°C for 1h, and subsequently alkylated with 10mM methyl methane-thiosulfonate (MMTS) for 10min. After cysteine blocking, each sample was diluted to 0.05% (w/v) SDS prior to trypsinization at 37°C for 16h. Following this, each tryptic digest was labeled for 1h with one of the four isobaric amine-reactive tags as follows: Tag\textsubscript{114} – 24h control; Tag\textsubscript{115} – 24h treated; Tag\textsubscript{116} – 36h treated; and Tag\textsubscript{117} – 48h treated samples. These four iTRAQ-derivatized samples were then pooled and passed through a strong cation exchange cartridge as recommended by the manufacturer (Applied Biosystems). This eluate (from the ion exchange step) was desalted using a Sep-Pak cartridge (Millipore), vacuum dried and reconstituted for 2-D LC.

**cICAT labeling.** The control and treated cells harvested from the 3 time-points (24h, 36h, and 48h) were lysed in 50mM Tris, 1.0% (w/v) SDS, pH 8.5, and boiled at 100°C for 10min. They were then subjected to centrifugation at 18,800g for 1h at 23°C to remove cell debris. cICAT labeling and processing of the samples followed standard protocols (Applied Biosystems). 100µg of protein from the control and butyrate-treated cell lysate of each time-point were each reduced with 1.25mM TCEP, and subsequently labeled with the respective isotopic light and heavy forms of the cICAT reagents, for 2h at 37°C. Each pair of heavy and light cICAT derivatized proteins from each time-point was then pooled and trypsinized at 37°C for 16h. Upon completion of *in-situ* digestion, the digested peptide mixture was cleaned up with
a strong-cation exchange cartridge, and then enriched with an avidin affinity cartridge. The cICAT-labeled peptides were then dried by speed vacuuming, dissolved in cleaving reagents and incubated at 37°C for 2h. After the removal of biotin, peptides were brought to dryness again before being reconstituted for 2-D LC.

**Two-Dimensional Liquid Chromatography (2-D LC) separation of labeled peptides.**

Each of the iTRAQ and cICAT-labeled peptide mixtures was separated using an Ultimate™ dual-gradient LC system (Dionex-LC Packings) equipped with a Probot™ MALDI spotting device. A 2-D LC separation was performed as follows: the labeled peptide mixture was dissolved in 2% acetonitrile (ACN) with 0.05% trifluoroacetic acid (TFA) and injected into a 0.3 × 150-mm strong cation-exchange (SCX) column (FUS-15-CP, Poros 10S) (Dionex-LC Packings) for the first dimensional separation. The mobile phase A and B were 5mM KH$_2$PO$_4$ buffer, pH 3, + 5% ACN and 5mM KH$_2$PO$_4$ buffer, pH 3, + 5% ACN + 500 mM KCl respectively. The flow rate was 6µl/min. 9 fractions were obtained using step gradients of mobile phase B: unbound, 0-5, 5-10, 10-15, 15-20, 20-30, 30-40, 40-50, 50-100%. The eluting fractions were captured alternatively onto two 0.3 × 1-mm trap column (3-µm C$_{18}$ PepMap™, 100 Å) (Dionex-LC Packings) and washed with 0.05% TFA followed by gradient elution in a 0.2 × 50-mm reverse-phase column (Monolithic PS-DVB) (Dionex-LC Packings). The mobile phase used for this second-dimensional separation were 2% ACN with 0.05% TFA (A) and 80% ACN with 0.04% TFA (B). The gradient elution step was 0-60% B in 15 min at a flow rate of 2.7µl/min. The LC fractions were mixed directly with MALDI matrix solution (7mg/ml α-cyano-4-hydroxycinnamic acid and 130µg/ml ammonium citrate in 75% ACN) at a flow rate of 5.4µl/min via a 25-ml mixing tee (Upchurch Scientific) before they were spotted onto a 192-well stainless
steel MALDI target plate (Applied Biosystems) using a Probot Micro Precision Fraction collector (Dionex-LC Packings), at a speed of 5 sec per well. 50 fmol of ACTH (18-39) peptide ($m/z = 2465.199$) was spiked into each well as internal standard.

**Mass spectrometry analysis.** The samples on the MALDI target plates were analyzed using a 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems). MS/MS analyses were performed using nitrogen at collision energy of 1 kV and a collision gas pressure of $1 \times 10^{-6}$ Torr. The GPS Explorer™ software v. 3.6 (Applied Biosystems) was used to create and search files with the MASCOT search engine (version 2.1; Matrix Science) for peptide and protein identifications in both the cICAT and iTRAQ-labeled samples. The International Protein Index (IPI) human database (Version 3.30, 67922 sequences) (20) was used for the search and this was restricted to tryptic peptides.

**iTRAQ-labeled samples.** One thousand shots were accumulated for each MS spectrum. For MS/MS, 6,000 shots were combined for each precursor ion with signal to noise (S/N) ratio greater or equal to 100. For precursors with S/N ratio between 50 and 100, 10,000 shots were acquired. The resolution used to select the parent ion was 200. No smoothing was applied before peak detection for both MS and MS/MS, and the peaks were deisotoped. For MS/MS, only the peaks from 60 Da to 20 Da below each precursor mass, and with S/N $\geq 10$ were selected. Peak density was limited to 30 peaks per 200 Da, and the maximum number of peaks was set to 125. Cysteine methanethiolation, N-terminal iTRAQ labeling, and iTRAQ labeled-lysine were selected as fixed modifications while methionine oxidation was considered as a variable modification. One missed cleavage was allowed. Precursor error tolerance was set to 100 ppm while MS/MS fragment error tolerance was set to 0.4 Da. Maximum peptide rank was set to
2. iTRAQ quantification was performed using the GPS Explorer™ software and normalized among samples. iTRAQ ratios were calculated based on the cluster areas of the iTRAQ reporter fragment peaks (114, 115, 116 and 117), and the ratios calculation included only peptides identified with C.I. % above cutoff thresholds as described below.

The average iTRAQ ratio and standard deviation (S.D.) were determined using the GPS Explorer™ software which was calculated using the following equations:

\[
R = e^{\left( \sum_{i=1}^{N} \frac{x_i}{N} \right)}
\]

where \( R = \) Average iTRAQ Ratio

\[ x_i = \text{natural log of iTRAQ Ratio of each iTRAQ pair} \]

\( N = \) the number of peptides with non-zero iTRAQ Ratio

\[
S.D. = e^{(\log R + \log S)} - e^{(\log R)} = e^{(\log R)} \times (e^{(\log S)} - 1)
\]

where \( S.D. = \) standard deviation of iTRAQ Ratio, and

\[
\log R = \frac{\sum_{i=1}^{N} x_i}{N}
\]

\( \log S = \) sd

\( R = \) Average iTRAQ Ratio
\[
sd = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \log R)^2}{N - 1}}
\]

where \(sd\) = iTRAQ Standard Deviation

\(X_i = \) natural log of iTRAQ Ratio of each peptide

In this work, 4 biological replicates of iTRAQ labeled samples were analyzed. Student’s \(t\)-test was performed, and the \(p\) values based on the iTRAQ ratios of peptides matched to each protein (48h time-point vs. control) were used to assess the significance of temporal differential expression. Proteins that have \(p\) value <0.05 in at least one dataset and shown consistent changes in all datasets were considered as significantly altered in the expression level.

To determine the cutoff threshold of fold changes for proteins with single peptide match, 2 equal amounts of tryptic digested six protein mixtures (Applied Biosystems) were labeled with iTRAQ reagent 114 and 117 respectively, and analyzed with 1-D LC MALDI-TOF/TOF MS (reverse phase liquid chromatography; similar to abovementioned). The standard deviation (S.D.) based on the ratios of all the identified peptides was 0.15, thus 1.3 (1 + 2 S.D.) was determined to be the significant cutoff threshold \((p < 0.05)\) for the up-regulated proteins, and reciprocally 0.77 was the cutoff threshold for the down-regulated proteins (Data was shown in the supplementary data). Similar cutoff threshold has been used in other iTRAQ studies (21, 22).

**cICAT-labeled samples.** For MS analysis, typically 1000 shots were accumulated for each sample well. MS/MS acquisition was performed in a result dependent manner. Only cICAT pairs
with normalized ratio (normalized against median ratio of all the cICAT pairs detected) greater than 40% were selected for fragmentation. Singletons were also selected as precursor ions. Stop conditions were implemented so that 3,000 to 6,000 shots were accumulated depending on the quality of the spectra. The resolution used for parent ion selection was 200. Peak processing and detection procedures were the same as above mentioned. Heavy and light cICAT-labeled cysteine, N-terminal acetylation and pyroglutamation (E & Q), and methionine oxidation were selected as variable modifications. One missed cleavage was allowed. Precursor error tolerance was set to 100 ppm and MS/MS fragment error tolerance to 0.3 Da. Maximum peptide rank was set to 5. cICAT quantification was performed using GPS Explorer™ software and normalized against median ratio obtained from all the cICAT peptide pairs detected in one sample. The ratios were calculated by comparison of the cluster area of the heavy-ICAT labeled peptide with that of the light-ICAT labeled peptide. Two equal amounts of tryptic digested BSA were labeled with heavy and light cICAT tags, and subjected to 1-D LC MALDI-TOF/TOF MS. The standard deviation (S.D.) based on the ratios of all the identified cICAT-labeled peptides was 0.12, thus 1.36 (1 + 3 S.D.) was determined as the significant cutoff threshold ($p < 0.01$) for the significantly up-regulated proteins, and reciprocally 0.74 was the cutoff threshold for the down-regulated proteins.

**Estimation of false positive rate to determine cut-off score.** In addition to the IPI human database, a randomized database (67922 sequences) generated using IPI human database Version 3.30 (generated using a Pearl script downloaded from http://www.matrixscience.com/help/decoy_help.html) was also used to search both the iTRAQ- and cICAT-labeled samples. The false positive rate was calculated by comparing the peptide hits obtained from these 2 databases at different ion score C.I. % (peptide). The minimum ion score
C.I. % was set such that no more than 5% false positive rate is achieved. Based on this cut-off threshold, all the proteins identified from the random database search were single-peptide matched. Hence, proteins identified from the human database that are matched to at least 2 peptides are statistically confident. For single-peptide matched proteins, only those with ion score C.I. % greater than the highest C.I. % attained from the random database search were selected. With these cut-off thresholds, we essentially achieved 0% false-positive identification rate at protein level. In addition, those single-peptide matched proteins must be identified based on peptide which has been detected several time in one run or in replicate runs. The minimum ion score thresholds that were used for each iTRAQ- and cICAT-labeled sample were shown in the supplementary data.

**Real-time PCR.** RNA was isolated from 2 batches of harvested HCT-116 cells using the RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Purified RNA was quantified by UV spectrophotometry (OD$_{260}$ of 1 = 40µg/ml) and assessed using denaturing agarose gel electrophoresis. MultiScribe™ Reverse Transcriptase (Applied Biosystems) was used to reverse transcribe RNA from each sample to cDNA, following the manufacturer’s protocol. Primers specific for each gene target was designed using Primer Express software (Applied Biosystems) and synthesized by 1st Base Pte Ltd (Singapore). BLAST searches for all primer sequences were performed to confirm gene specificity. For quantification of each gene in the samples, amplification was performed in triplicates with SYBR Green PCR Master Mix (Applied Biosystems) on the ABI PRISM 7000 Sequence Detection System instrument, according to the manufacturer’s instructions. Non-template controls were included for each run. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous
control reference for normalization. Thermal cycling parameters were as follows: denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

2-Dimensional Gel Electrophoresis (2-DE). 2-DE was performed as previously described (17). Briefly, harvested cells were lysed in the extraction buffer and clarified with centrifugation. 10µg of each sample was then loaded onto rehydrated 7cm 3-10 NL IPG strips and separated on the IPGphor unit (GE Healthcare) using the following parameters: (i) 100 V, 50 Vhr; (ii) 200 V, 100 Vhr; (iii) 500 V, 250 Vhr; (iv) 1000 V, 500 Vhr; (v) 1000-8000 V, 2250 Vhr, and (vi) 8000 V, 12000 Vhr. A two-step equilibration procedure using dithiothreitol (DTT) and iodoacetamide (IAA) was used to reduce and alkylate the separated proteins in the IPG strips respectively, before the second dimensional SDS-PAGE step.

Western blot. Equal aliquots of proteins extracted from both control and treated cells of each time-point were resolved in 1-D SDS-PAGE. Upon completion of electrophoresis, the proteins were electroblotted onto nitrocellulose membranes (Bio-Rad). The blots were then blocked using 5% (w/v) nonfat dry milk in TBS with 0.1% Tween 20 (TBS-T) overnight prior to immunoprobing with antibodies diluted in TBS-T with 1% (w/v) milk for 1h each. The membranes were incubated with rabbit anti-GAPDH (1:200) from Santa Cruz, mouse anti-HSP 90-beta (1:1,000) from Stressgen, mouse anti-Gelactin-1 (1:500), mouse anti-AKAP12 (1:500), mouse anti-SEC22b (1:750), or mouse anti-COX VIb (1:750) from Abnova. HRP-conjugated anti-rabbit IgG (1:2,500) from Santa Cruz, HRP-conjugated anti-mouse IgG (1:5,000) from GE Healthcare or HRP-conjugated anti-mouse IgM (1:5,000) from Pierce were used as secondary antibodies. 3 washes in TBS-T were carried out between each antibody incubation. Subsequent
visualization was performed using enhanced chemiluminescence (ECL) (GE Healthcare) with GAPDH levels as the loading control.

Results and Discussion

Protein identification from iTRAQ- and ICAT-labeled peptides.

783 unique proteins were identified from a total of 3,116 tryptic peptides for the iTRAQ-labeled samples. On the other hand, 137 unique proteins were identified from a total of 241 peptides obtained from cICAT (see supplementary data for the lists of iTRAQ- and ICAT-labeled proteins that showed temporal differential expression after butyrate treatment). Due to the difference in labeling chemistry, the result obtained from the cICAT approach complements the iTRAQ data. Recently, quantitative proteomics incorporating stable isotope tagging such as post-isolation labeling using ICAT or iTRAQ was demonstrated to be a complementary strategy to 2-DE (23, 24). Most notably, a comparative study of these three proteomics methods found limited overlapping proteins between them, and iTRAQ was considered to be the more sensitive technology as compared to ICAT and 2-D DIGE (25). This underscored the importance of using various technology platforms for a more comprehensive proteomics study of complex samples.

Interestingly, a subset of proteins found in this study had also been identified in our previous work using 2-D DIGE (17), and they showed regulation in the similar manner by butyrate treatment. Such proteins include cytoskeletal 8, ornithine aminotransferase, cytochrome c oxidase polypeptide VIb and Tu elongation factor.

Temporal analysis of proteins following butyrate treatment
From the list of differentially expressed proteins obtained from this temporal study, proteins that exhibited progressive up- or down-regulation were clustered into groups on the bases of their biological functions. They could be grouped into four cellular processes, \textit{viz.}, A) growth arrest, B) apoptosis, C) metabolism, and D) metastasis (Figure 1, also see supplementary data for the complete list of differentially expressed proteins). Subsequently, some of these protein candidates were validated using quantitative real-time PCR and/or western blotting. These results are shown in Fig. 2 and 3, and they are in accord with the proteomic results.

An overview of the temporal anti-cancer effects of butyrate treatment on the various cellular processes is shown in Fig. 4. This data is obtained from the iTRAQ ratios of the proteins grouped under each cellular processes at each time point. As seen here and discussed further below, our temporal analysis showed that butyrate induced a blockage of cell cycle progression as an early event (24h) whereas the anti-metastasis effect was most apparent at the later stage (48h) of treatment.

\textbf{Temporal Regulation of the Cellular Processes and Pathways induced by Butyrate.}

This study has clearly identified clusters of proteins in pathways that correlate protein expression changes with the induction of anti-cancer effects. The synergistic influence of each cluster of proteins results in the overall phenotypic response to butyrate. On the bases of these observations, we proposed a model to illustrate the integrated cellular mechanism initiated by butyrate in colorectal cancer cells (Figure 5).

\textbf{Cluster A: Growth arrest}
**Cell cycle progression.** Butyrate regulates several cell cycle genes including c-myc, p16 and p21 (14). Among the list of down-regulated proteins identified here, several function in nucleotides biosynthesis, cell cycle progression and cellular proliferation (Figure 1). These proteins include DNA replication licensing factor MCM7, ran-specific GTPase-activating protein, caprin 1 and nucleosome assembly protein 1-like 1 (the latter two proteins were verified by real-time-PCR). The down-regulation of these cell cycle regulatory proteins is in concordance with the inhibition of DNA replication, hindrance of cell division and hence blockage of cell cycle progression by butyrate, as represented in Cluster A of our proposed model (Figure 5). Our temporal analysis showed that butyrate induced an early reduction in the expression of these proteins, which plateaued after the 36h time-point (Figure 4).

**Signaling.** A-kinase anchoring protein 12, a scaffold protein for kinases (26) that possesses tumour suppressor activity, was found to be dramatically down-regulated in both iTRAQ and cICAT data (also verified by real-time PCR; Figure 2). Multiple intracellular kinases in the oncogenic or survival signaling pathways have been illustrated to be key players in butyrate actions (27, 28). Western blotting using monoclonal antibody against the C-terminal of AKAP12 showed decreased expression of the full length protein and an appearance of a truncated isoform at \( M_r \approx 40\text{kDa} \) over time (Figure 3). Peptides from the MS/MS spectra of AKAP12 matched to only sequences from the N-terminal part of the protein, supporting the decreased presence of native AKAP12 which was verified in the western blot band at \( \approx 200\text{kDa} \). The 2-DE western blot clearly verified the increased abundance of the fragmented protein of \( \approx 40\text{kDa} \) (Figure 3). This preliminary data may indicate that targeting of kinases by AKAP12 may be regulated by butyrate, thus affecting downstream growth-associated signaling cascades.
On the other hand, the N-terminal fragment of AKAP12 may contribute to the tumour suppressor property of this protein. These await further investigations.

**Cluster B: Apoptosis**

As demonstrated in Figure 5, the proteins in Cluster B function as tumour suppressors, heat shock proteins and chaperones, players in the oxidative phosphorylation pathway, or ubiquitination-proteasome pathway respectively. The temporal changes in expression of these proteins contribute to the initiation of apoptosis by butyrate in HCT-116 cells.

**Tumour suppressors.** As shown in Figure 1, tumour suppressors, such as galectin-1, metallothionein-1X, prohibitin-2, and ras-related protein Rap-1A, displayed a temporal increase in expression level upon butyrate treatment in this study. These proteins contribute to tumour growth suppression by butyrate. For example, galectins are multifunctional β-galactoside lectins with roles including cell adhesion, growth regulation, invasion, and apoptosis (29). The identification of up-regulated galectin-1 here (validated with western blot in Figure 3) corroborated with previous work that showed its association with butyrate’s actions (30, 31). We also found metallothionein-1X was markedly up-regulated by butyrate and this was confirmed by real-time PCR (Figure 2). The regulation of metallothioneins is not uniform in all tumors. For instance, this protein was over-expressed in bladder cancer but down-regulated in advanced prostate cancer (32, 33). Although other metallothioneins have been found to be up-regulated by butyrate in a paradigm of increased resistance to toxic metals in tetracarcinoma and hepatoma cells (34, 35), this is the first report on the regulation of metallothioneins by butyrate in colorectal cancer cells. Metallothioneins have a high metal binding affinity for metal homeostasis and detoxification. Exposure to metals such as chromium, nickel, iron, copper and
manganese has been shown to promote carcinogenesis. Thus, the increased expression of metallothionein by butyrate may relate to the regulation of metals associated with colorectal carcinogenesis.

Similarly, our results also showed that voltage-dependent anion-selective channel protein 1 (VDAC1) and ADP/ATP translocase 2 (ANT2) were found to be concurrently up-regulated by butyrate. Their expression levels were shown to increase, particularly after the 36h time-point (Figures 1 and 4). These proteins are candidate regulators of cytochrome c release via the mitochondrial transition pore (MTP) for activation of apoptotic cascades. Mitochondria play a pivotal role in apoptosis (36, 37), and the release of proapoptotic proteins, like cytochrome c, apoptosis-inducing factor, and Smac/Diablo from mitochondria is crucial in mediating apoptosis by chemotherapeutic agents. The extrinsic apoptotic pathway mediated by cytochrome c release was activated by butyrate treatment (38). VDAC1 has an increased expression level as seen from the cICAT results. It plays an essential role in the translocation of apocytochrome c for the activation of downstream caspases. Over-expression of this mitochondrial protein has been found to induce cell death (39). ANT2 catalyze the exchange of ADP/ATP across the mitochondrial membrane, and have been implicated in apoptosis mediated through the mitochondrial transition pore as well (40). The increased expression of these regulators of MTP may contribute to the activation of cytochrome c mediated apoptotic cascades by butyrate. The measurement of these tumour suppressors in cancer cells could thus serve as monitors of the efficacy of pro-apoptotic drug treatment.

**Oxidative phosphorylation.** Our results clearly reflected a trend of increased expression of the electron transport chain (ETC) complexes (Figure 1). The rise in expression levels of these proteins were further increased after 36h of butyrate treatment. Amongst the proteins, differential
expression of cytochrome c oxidase Va and VIb were verified with real-time-PCR (Figure 2). Cytochrome c oxidase VIb was also reported to be up-regulated by butyrate in our previous work (17), and western blotting confirmed the result here (Figure 3). The increased expression of proteins in the oxidative phosphorylation pathway may relate to the enhanced mitochondrial activity by butyrate, and subsequent growth arrest and apoptosis in the colonic epithelial cells (41). Our study has also shown up-regulation of ATP synthase subunit of Complex V upon butyrate treatment. ATP synthase was down-regulated in colorectal carcinoma as an avoidance mechanism towards reactive oxygen species (ROS) – mediated cell death (42). The study by Giardina et al. (43) has shown a role for butyrate influence on ROS generation in colon carcinogenesis. The changes in the expression levels of ETC complexes, such as complex I, II, IV and V, as seen here may result in unstable mitochondrial membrane potential and an increase in ROS production. Hence, in addition to possible generation of ATP from the enhanced oxidative phosphorylation for the energy-dependent apoptosis, cytotoxic mitochondrial ROS production could sensitize butyrate-treated cells to oxidative stress-mediated cell death (Schematized in Figure 5, Cluster B).

**Heat shock proteins (HSPs) and Chaperones.** A temporal decrease in the expression of chaperones, such as heat shock 27kDa protein, heat shock protein HSP90, heat shock cognate 71kDa protein and thioredoxin, was detected in butyrate-treated HCT-116 cells (Figure 1). The degree of down-regulation was shown to be reduced after the 36h time-point. HSPs act as molecular chaperones, thus playing an indispensable role in defense against cellular stress such as chemotherapy-induced apoptosis (44). HSP90, one of the down-regulated chaperones identified here (confirmed with real-time-PCR and western blot), was advocated as a novel anticancer target (45) and its inhibitor 17-allylaminogeldanamycin (17AAG) is currently in anti-
cancer clinical trial. HSP90 is responsible for maintaining the stability of many oncogenic proteins with biological functions in cellular proliferation and apoptosis. HSP90 is known to be dysfunctional in tumours (46, 47), and was detected to be up-regulated in transformed cells. Inhibitors of this anti-apoptotic protein triggered cancer cell death synergistically with butyrate treatment (48). The reduced expression of chaperones as shown here will deter proper protein folding leading to protein aggregation, ultimately resulting in cell death in cancer cells.

**Ubiquitination-Proteasome pathway.** Proteasome activator subunit 2, ubiquitin-activating enzyme E1 and F-box only protein 2 are some of the proteins in the ubiquitination-proteasome pathway that were also noted to be differentially regulated by butyrate, as shown in our results (Figure 1). Degradation of proteins via the ATP/ubiquitin-dependent pathway mediates apoptosis (49). Targets of the 26S proteasome include proteins in heat shock response and cell cycle control (50, 51); both systems were found to be down-regulated in this study (Figures 1 and 4). The butyrate-induced apoptotic cascades are associated with the ubiquitin-degradation system, and inhibitors of the proteasome act synergistically with butyrate in anti-carcinogenic therapy. In support of this, Pei *et al.* (52) found that the simultaneous application of a proteasome inhibitor and butyrate could induce apoptosis. Both Yu *et al.* (53) and Giuliano *et al.* (54) showed similar synergistic effects between proteasome activity and butyrate. Hence, butyrate-regulated ubiquitination-proteasome pathway would affect the levels of survival- and apoptosis-related proteins in cancer cells.

**Cluster C: Metabolism**

Our data identified a repertoire of biosynthetic enzymes, including those involved in the Krebs cycle and pentose phosphate pathway, to be up-regulated by butyrate in a time-dependent
manner (Figure 1). The change in the expression levels for most of these proteins was shown to be more pronounced after 36h of treatment. Examples of these metabolic enzymes were malate dehydrogenase, oxoglutarate (alpha-ketoglutarate) dehydrogenase, transaldolase and transketolase. This suggested that butyrate altered the metabolic machinery of HCT-116 cells. Most tumours including CRC depend on the enhanced glycolysis instead of oxidative phosphorylation for ATP production, even in the presence of oxygen, a phenomenon known as the “Warburg effect” (55). The metabolic enzymes found to be up-regulated by butyrate in this study are involved in various glucose metabolic pathways which thus promote glucose metabolism. However, unlike other metabolic enzymes, alpha-enolase was shown to be down-regulated by butyrate. This may retard the rate of glycolysis since enolase catalyzes the formation of phosphoenolpyruvate, a precursor of glycolytic end-product pyruvate. Furthermore, several enzymes functioning in the oxidative phosphorylation pathway were up-regulated by butyrate (as discussed earlier).

Butyrate demonstrates phenotypical specificity whereby it causes growth arrest followed by differentiation and/or apoptosis in carcinoma cells but promotes proliferation in normal cells (56). Colonic carcinoma cells derive energy via metabolism of glucose whereas normal colonic epithelial cells oxidize butyrate as the key fuel source for cellular proliferation (57–59). Butyrate has been reported to induce apoptosis in the presence of glucose and pyruvate but promote growth in the absence of these alternative energy sources (60). Herein, butyrate altered the metabolic profile of cancer cells, resulting from an enhanced expression of several metabolic enzymes. Metabolism of other energy sources as fuel thus avails butyrate to effect its anti-cancer actions in HCT-116 cells.
In addition, proteins functioning in amino acids and lipid/cholesterol metabolic pathways, such as ornithine aminotransferase, asparagine synthetase, argininosuccinate synthase, delta 1-pyrroline-5-carboxylate synthetase and enoyl-CoA hydratase, were up-regulated in this study. Leschelle et al. (61) and Tabuchi et al. (62) have demonstrated stimulated lipogenesis by butyrate. Ruemmele et al. (63) and Della Ragione et al. (64) found that inhibiting protein synthesis by cycloheximide blocked butyrate-induced apoptosis. In this work, vesicular transport proteins, which function in protein synthesis such as vesicle trafficking protein SEC22b (verified by real-time PCR and western blot), clathrin heavy chain 1 and NAPB protein, were identified to be up-regulated. These pathways were grouped under Cluster C in the proposed model (Figure 5).

**Cluster D: Metastasis**

**Cytoskeleton-associated proteins.** In correlation to previous reports on cytoskeletal organization of cancer cells (65, 66), the data here showed increased expression of various cytoskeleton-related proteins by butyrate (Figure 1). The overall increase in the expression level of these proteins was higher after the 36h time-point. The concerted temporal up-regulation of these proteins such as cytoskeletal 18, cytoskeletal 19, epiplakin and filamins may lead to a strengthened cytoskeletal scaffold and reduced metastasis potential of carcinoma cells (Cluster D in Figure 5). Several of these identified proteins function as crosslinkers in the intermediate filament network, modulating cell adhesion, motility and invasiveness. Real-time PCR was conducted for cytoskeletal 19 (Figure 2). LIM domain and actin-binding protein, also known as the elevated expression of epithelial protein lost in neoplasm (EPLIN), identified by cICAT, diminish the invasiveness of cancer cells. EPLIN is a cytoskeleton-associated protein whose down-regulation in cancer cells may facilitate motility of these cancer cells (67). Our results
showed that the anti-metastasis effect was induced as a later event, after growth inhibition and apoptosis (Figures 1 and 4). The anti-metastasis effect shown here corresponds to the \textit{in vivo} study done by Velazquez \textit{et al.} (68) that demonstrated inhibition of seeding and growth of colorectal metastases to the liver by intravenous infusion of butyrate in mice. Butyrate is currently evaluated in clinical trials and has shown optimistic results (69).

\textbf{Conclusion}

A global quantitative proteomics approach was utilized in this analysis of the temporal effects of butyrate in HCT-116 cells. Differentially expressed proteins identified from this study were grouped according to their biochemical functions and a model depicting the integrated cellular processes initiated by butyrate was proposed (Figure 5). The temporal and synergistic effects of each pathway would lead to the anti-proliferative and pro-apoptotic properties of butyrate.

As shown in Figure 4, our study demonstrated that butyrate reduced expression of cell cycle regulatory proteins and nucleotides biosynthesis proteins which led to growth arrest at the early stage and tapered after the 36h time-point. The regulation of HSPs and ubiquitination-proteasome pathway by butyrate was less significant. On the other hand, the expression levels of proteins that function in oxidative phosphorylation, metabolism, or as tumour suppressors increases on a temporal basis with a similar trend. Moreover, there is a greater increase in their expression levels after the 36h time-point. The synergistic up-regulation of these proteins induces apoptosis in HCT-116 cells. The anti-metastasis effect of butyrate was most significant, and strongly accentuated at the late phase of treatment.
These signature clusters of butyrate-regulated pathways could serve as potential therapeutic targets or proteomics markers to assess drug candidates’ efficacy or toxicity. Our data clearly showed that in addition to targeting proteins involved in cell cycle blockage, apoptotic and anti-metastatic pathways, butyrate also alters the metabolic profile of the cancer cells to induce its anti-cancer effects. A better understanding of the mechanism whereby butyrate mediates its therapeutic actions would certainly aid in the design of better therapeutic intervention. Thus, multi-drugs regimen(s) that have synergistic effects on these clusters of pathways may be a promising pharmacological strategy for chemoprevention of colorectal cancer.

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**Supplemental Data**

The supplementary data include the minimum ion score thresholds used, determination of cutoff threshold of fold changes for proteins with single peptide match, and the list of iTRAQ- and cICAT-labeled proteins that showed temporal differential expression after butyrate treatment. The ratio and sequence of peptides that are matched to the proteins (for both ≥2 peptides and single-peptide based identifications), and the MS/MS spectra for single-peptide based protein identifications are also included.

**Figure Legends**

**Figure 1. Identification of protein clusters based on biological functions that showed similar trends of differential expression over time.** These proteins exhibit progressive up- or down-regulation on a temporal basis and were clustered into groups of certain cellular processes modulated by butyrate: A) cell cycle progression B) apoptosis (B1-tumour suppressors, B2-oxidative phosphorylation, B3-HSPs and chaperones, B4-ubiquitination-proteasome pathway), C) metabolism, and D) metastasis.
Figure 2. Validation of the iTRAQ results on selected proteins using real-time PCR. The results verified differential regulation of these proteins upon butyrate treatment. Fold-change ratio assessed by real-time-PCR was expressed as mean values ± standard error (S.E.) of 2 batches of cells performed in triplicates.

Figure 3. Western blots of proteins identified to have differential expression from iTRAQ data. 3a. Western blot confirmed differential expression of these proteins. GAPDH was used as the loading control. For AKAP12, decreased expression of full length protein (~200kDa) was detected but an increased presence of a protein fragment ~40kDa was seen over time. 3b. 2-DE (pH 3-10) western blot for AKAP12 was performed to confirm the increased expression of the fragment protein at ~40kDa (Circled in Figure 3b).

Figure 4. An overview of the temporal effects of butyrate treatment on the various cellular processes. The differential regulation of the proteins from each cellular process was summarized to illustrate the overall temporal effects of butyrate treatment on HCT-116 cells.

Figure 5. A model depicting pathways initiated by butyrate in mediating growth arrest and apoptosis in HCT-116 cells was proposed. (A) Reduced expression of cell cycle regulatory proteins and nucleotides biosynthesis proteins led to growth arrest induced by butyrate. (B) Butyrate increased the expression of tumour suppressors, proteins associated with mitochondrial transition pore (MTP) for the translocation of cytochrome c, and modulated the expression of chaperones and proteasome pathways, resulting in the activation of apoptosis cascades. (C)
Metabolic machinery of the cells was altered with an increased expression of several metabolic enzymes. (D) Expression of cytoskeleton-associated proteins was increased to strengthen cytoskeletal scaffold and lower the metastasis potential of HCT-116 cells.
Cluster B2: Oxidative Phosphorylation

Cluster B3: HSPs and Chaperones

- NDUFA10 NADH dehydrogenase ubiquinone 1 alpha subcomplex
- SDHA Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
- COX5A Cytochrome c oxidase subunit 5A, mitochondrial precursor
- COX6B1 Cytochrome c oxidase subunit VIb isoform 1
- ATP5B ATP synthase subunit beta, mitochondrial precursor
- ATP5H Isoform 1 of ATP synthase D chain, mitochondrial

- HSPB1 Heat-shock protein beta-1
- HSPA4 Heat shock 70 kDa protein 4
- HSPA8 Isoform 2 of Heat shock cognate 71 kDa protein
- HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein
- HSP90AA1 Heat shock protein HSP 90-alpha
- HSP90AB1 Heat shock protein 90Bb
- HSP90AB1 Heat shock protein 90Bd
- HSP90AB1 Heat shock protein HSP 90-beta
- C6orf115 similar to Protein C6orf115
- CCT2 T-complex protein 1 subunit beta
- PRDX5 Isoform Mitochondrial of Peroxiredoxin-5, mitochondrial precursor
- PRDX2 Peroxiredoxin-2
**Cluster B4: Ubiquitination-Proteasome Pathway**

- PSME2 proteasome activator subunit 2
- UBE1 Ubiquitin-activating enzyme E1
- FBXO2 F-box only protein 2

**Cluster C: Metabolism**

- MDH2 Malate dehydrogenase, mitochondrial precursor
- OGDH oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) isoform 1
- IDH1 Isocitrate dehydrogenase [NADP] cytoplasmic
- C12orf5 Uncharacterized protein C12orf5
- TALDO1 similar to transaldolase 1
- ENO1 Isoform MBP-1 of Alpha-enolase
- ENO1 Isoform alpha-enolase of Alpha-enolase
- OAT Ornithine aminotransferase, mitochondrial precursor
- ALNS Asparagine synthetase
- ASS1 Argininosuccinate synthase
- ALDH18A1 Isoform Short of Delta 1-pyrroline-5-carboxylate synthetase
- ECHS1 Enoyl-CoA hydratase, mitochondrial precursor
- DBI Isoform 2 of Acyl-CoA-binding protein
- CYB5R3 Isoform 1 of NADH-cytochrome b5 reductase 3
- CKB Creatine kinase B-type
Figure 1. Identification of protein clusters based on biological functions that showed similar trends of differential expression over time. These proteins exhibit progressive up- or down-regulation on a temporal basis and were clustered into groups of certain cellular processes modulated by butyrate: A) cell cycle progression B) apoptosis (B1-tumour suppressors, B2-oxidative phosphorylation, B3-HSPs and chaperones, B4-ubiquitination-proteasome pathway), C) metabolism, and D) metastasis.
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