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Target Identification of Grape Seed Extract in Colorectal Cancer using Drug Affinity Responsive Target Stability (DARTS) Technique: Role of Endoplasmic Reticulum Stress Response Proteins

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Abstract

Various natural agents, including grape seed extract (GSE), have shown considerable chemopreventive and anti-cancer efficacy against different cancers in pre-clinical studies; however, their specific protein targets are largely unknown and thus, their clinical usefulness is marred by limited scientific evidences about their direct cellular targets. Accordingly, herein, employing, for the first time, the recently developed drug affinity responsive target stability (DARTS) technique, we aimed to profile the potential protein targets of GSE in human colorectal cancer (CRC) cells. Unlike other methods, which can cause chemical alteration of the drug components to allow for detection, this approach relies on the fact that a drug bound protein may become less susceptible to proteolysis and hence the enriched proteins can be detected by Mass Spectroscopy methods. Our results, utilizing the DARTS technique followed by examination of the spectral output by LC/MS and the MASCOT data, revealed that GSE targets endoplasmic reticulum (ER) stress response proteins resulting in overall down regulation of proteins involved in translation and that GSE also causes oxidative protein modifications, specifically on methionine amino acids residues on its protein targets. Corroborating these findings, mechanistic studies revealed that GSE indeed caused ER stress and strongly inhibited PI3k-Akt-mTOR pathway for

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CONFLICTS OF INTEREST

No potential conflicts of interest were identified by any authors of this manuscript.

its biological effects in CRC cells. Furthermore, bioenergetics studies indicated that GSE also interferes with glycolysis and mitochondrial metabolism in CRC cells. Together, the present study identifying GSE molecular targets in CRC cells, combined with its efficacy in vast pre-clinical CRC models, further supports its usefulness for CRC prevention and treatment.

Keywords

chemoprevention; colorectal cancer; Grape seed extract; DARTS

INTRODUCTION

According to the American Cancer Society, one out of four deaths in the United States is cancer-associated, with colorectal cancer (CRC) being the third leading cause of these deaths; over 50,800 deaths in 2013 were estimated to occur as a result of advanced CRC progression [1, 2]. Several strategies to screen CRC are utilized, but neither they have reduced disease incidence nor the related mortality [1-3]. In this regard, there is a growing interest to identify preventive strategies which focus on natural small-molecule drugs or nutraceuticals that can be administered on a daily basis for the treatment and prevention of CRC [4, 5]. Many of these small-molecule drugs form a major part of today's medicines; however, identification of specific protein targets of these small molecules remains a key challenge in creating effective clinical therapies [6-9]. Examining drug-protein interactions, prior to pre-clinical efficacy studies, allows scientist to effectively screen for the best small molecule candidates and to further predict any associated toxicity with the drug administration [7-9]. Amongst the various natural agents screened, grape seed extract (GSE) is one such non-toxic chemopreventive agent which has demonstrated anticancer efficacy in various pre-clinical *in vitro* and *in vivo* models of prostate, lung, breast, bladder and colon cancers [4, 10-21]. GSE contains proanthocyanidins [a mix of dimers, trimers and other oligomers (procyanidins) of catechin and epicatechin and their gallate derivatives], which are also widely distributed throughout the plant kingdom and are present in high quantities within the seeds of the grapes [12, 22-24]. Whereas molecular mechanisms of GSE are being extensively investigated, its direct protein targets are yet to be identified. The current methods to identify protein targets of polyphenolic mixtures, such as GSE, require alteration in the chemical compounds, to allow for detection; these affinity-based methods include: matrix-based affinity detection; genetic yeast three-hybrid and phage cloning [7]. Additionally, when considering a complete cellular system, which is composed of numerous chemical compounds and various proteins, there needs to be sensitive affinity-based techniques to identify and quantify these agents-protein interactions [7-9]. Current affinity-based techniques that are utilized to characterize complex chemical protein mixtures are limited by the need to modify the small molecule [7-9]; however, an alteration in the chemical compounds is not desirable, due to the potential structure alterations which can alter potential protein binding. An alternative approach is an indirect non-affinity technique; however, these techniques depend on the ability of the small molecule to induce the specific cellular or biochemical readout [7-9]. To overcome this obstacle, recently, there has been the development of a simple approach that analyzes the direct binding of drug to its specific targets; this technique is a universal applicable target identification approach [7-9]. The drug

affinity responsive target stability (DARTS) technique is a new method that like affinity methods relies on the affinity of the small molecule to bind to the target protein [7-9]. We anticipated that this target affinity would allow the identification of the direct GSE target proteins; notably, the key advantage of DARTS over current affinity based technique, is that it does not require chemical alteration of the components of GSE. DARTS allows for identification of potential target proteins that can then be further validated through molecular and biochemical techniques [7-9]. The theory behind the DARTS technique is that a given cellular protein may become less susceptible to proteolysis, when it is bound to drug, versus drug-free protein [7-9]. Taken together, in the present study, we aimed to identify potential protein targets of GSE, via the DARTS technique, in human CRC cell with the aim that this would help in the development of effective, long-term treatment and prevention approaches for CRC with GSE. The outcomes of these studies were further confirmed and supported by additional mechanistic studies focusing on associated signaling pathways and biological events.

Materials and Methods

Reagents

The composition of the standardized GSE preparation (Kikkoman Corp., Nado City, Japan) is listed as: 89.3% procyanidins, 6.6% monomeric flavanols, 2.24% moisture content, 1.06% of protein, and 0.8% of ash [10, 11, 25]. Dimethyl Sulfoxide (DMSO), oligomycin, antimycin A, 2-deoxyglucose (2-DG), carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP) were from Sigma Chemical Co. (St. Louis, MO). ER-ID™ Red dye (endoplasmic reticulum selective dye) was from Enzo life sciences (Farmingdale, NY). Primary antibodies anti-GRP78, anti-calnexin, anti-IRE1 α , anti-ATF6 α , anti-eIF2 α , anti-integrin β 1, anti-phospho IRS (Tyr 612), anti-phospho Akt (Ser 473), anti-phospho mTOR (Ser 2448), anti-phospho ERK(1/2) (Th202/Tyr204), anti-phospho P70S6K (Thr 389), anti-phospho 4E-BP1 (Thr 37/46), anti-phospho 4E-BP1 (Ser 65), anti-phospho EIF-4G (Ser 1108), anti-phospho EIF-4B (Ser422), anti-phospho EEF2 (Thr 56) and the respective total protein antibodies were from Cell Signaling Technology; anti-CK-1 and anti-DHE3 antibodies were from Abcam (Cambridge, MA); and anti- β -actin antibody was from Sigma (St. Louis, MO). Anti-mouse and anti-rabbit horseradish peroxidase (HRP) secondary antibodies were purchased from Invitrogen and Cell Signaling Technology, respectively.

Cell lines and Treatments

Authentication of human CRC cell lines SW480 and HCT116 cells (American Type Culture Collection, Manassas, VA), and SW620 cells (gift from Dr. Pamela Rice, University of Colorado, Denver) was done by polymorphic short tandem repeat profiling. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere in DMEM (HCT116) and RMPI (SW480 and SW620) media supplemented with 10% FBS and 1% penicillin/streptomycin. For serum starvation experiments, at 60% confluency, cells were serum starved (SS) for 24 h, treated with different concentrations of GSE and after 6h, stimulated with IGF-1 (50 ng/mL) for 15 min, and harvested as described previously [26].

DARTS with Complex Protein Mixtures

Human CRC cells were plated at a density of 5,000/cm² and under standard culture conditions were treated either with varying concentrations of GSE (25-100 µg/mL) in DMSO or with DMSO alone (control). At the end of 3h (treatment time), cell lysates were prepared in non-denaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, 5 units/mL aprotinin]. Bradford analysis was performed to ensure equal amount of protein lysate per sample. To prevent premature protein degradation in cell lysates, all steps were performed on ice. For proteolysis, each cellular lysate sample was proteolysed at room temperature for 5 min with Pronase. A 1:25, 1:50 and 1:100 ratios of Pronase versus cell lysates protein concentration were used for proteolysis, as described previously [7-9]. Proteolysis was stopped by addition of 0.5 M EDTA (pH 8.0) to each sample at a ratio of 1:10, mixed well, and then placed on ice. Samples were loaded on SDS-PAGE and run in 1 × SDS running buffer. At the end, gels were stained with Coomassie, then destained, and finally stored at 4°C in 5% acetic acid solution in glass containers until further in-gel digestion steps were performed.

In Gel-digest and LC/MS Analysis

Specific gel bands that were seen in treatment samples compared to controls were cut out and processed with trypsin digestion for mass spectrometry analysis as per the published protocol with some modifications (Fig. 1) [7-9]. Briefly, gel pieces were placed on a clean plate, each band was excised with a clean new scalpel; the larger pieces were cut to 1×1mm pieces. The gel pieces were washed twice with destaining buffer [25mM NH₄HCO₃ / 50% ethanol (EtOH)] and incubated for 20 min at 25°C, followed by dehydration of the gel pieces in 100% Acetonitrile (AcN) for 10 min at 25°C; the process was repeated until gel pieces were dry. Gel pieces were further dried in a speed-vac for 5 min and then re-hydrated in reduction buffer [10mM dithiothreitol (DTT) in 50 mM NH₄HCO₃, solution is further diluted to 1M DTT] and incubated for 60 min at 56°C. Alkylation buffer was then added (55mM iodoacetamide in 50mM NH₄HCO₃) and samples were incubated in the dark for 45 min at 25°C, followed by a gel wash with digestion buffer for 20 min at 25°C. These gel pieces were dehydrated again with 100% AcN for 10 min at 25°C, followed by drying again for 5 min in the speed-vac. Gel pieces were then again rehydrated in trypsin at 37°C for ~20 min and then further incubated for digestion with trypsin overnight at 37°C. From the gel matrix, the peptides were extracted by incubating gel pieces with extraction buffer [3% trifluoroacetic acid (TFA)/ 30% AcN], followed by gel dehydration and removal of the supernatant [7-9]. The digested proteins were loaded onto a Magic AQ C18 reverse phase nano column (Bruker) using a nano-Advance autosampler and nano flow UPLC (Bruker). Mobile phases consisted of H₂O + 0.1% formic acid (A) and 89.95% acetonitrile, 9.95% H₂O, 0.1% formic acid (B). Peptides were chromatographically separated at a flow rate of 500nl/min using a gradient of 5-45% B over 30 minutes followed by a column wash at 95% B for 5 min. For MS/MS analysis of the eluting peptides, an Amazon Speed ion trap equipped with a Captive Spray source (Bruker) was used. Proteinscape software (Bruker) was used to submit the data to MASCOT v.2.4 (Mass Spectrometry data analyses software) for database searching and the Percolator algorithm rescored peptide and protein matches.

Western Blot Analysis

The protein concentration of lysates was estimated using Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Samples were run on SDS-PAGE (8-16% tris-glycine gels) and blotted onto nitrocellulose membranes. Specific primary antibodies and peroxidase-conjugated appropriate secondary antibodies were used to probe the membranes using the ECL detection system from GE Healthcare (Buckinghamshire, UK). In certain cases, membranes were also probed with appropriate secondary IR Dye-tagged antibodies. These western blot membranes were also stripped and used again to re-probe for other proteins or loading control (β -actin); however, only representative loading control blots are shown.

Immunofluorescence

The Endoplasmic Reticulum (ER) staining was performed by using the ER-ID Red assay kit (Enzo life sciences). Cells were plated and cultured to 50-60% confluency on cover slips in six well culture plates. At the end of GSE treatment, the cells were fixed with 3.7% buffered formalin. The fixed cells were permeabilized with 0.3% Triton-X 100 and exposed to ER-ID Red dye (1: 100 dilution in PBS) for 20 min at 37° C. They were then counterstained with DAPI in the mounting medium, cell images with red stained ER were captured at 60X $\times$ 2.3 magnification on a Nikon D Eclipse C1 confocal microscope.

Extracellular Flux Analyzer Assays

XF24 Extracellular Flux Analyzer from Seahorse Bioscience (North Billerica, MA) was utilized to detect both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as an indicator of mitochondrial respiration and active glycolysis, respectively, in SW480 and HCT116 cells. Briefly, cells were plated in XF 24-well cell culture micro plates at a density of 4.0×10^4 cells/well using regular growth medium, and incubated at 37°C/5 % CO₂ for 24 h, followed by GSE treatment for 9 h. Upon termination of the experiment (9 h post GSE), cells were washed twice with XF24 assay medium (RPMI and DMEM media without FBS, for SW480 and HCT116 cells, respectively), and then incubated further in XF24 assay medium in the absence of CO₂ for 30 min before proceeding to measure real-time OCR and ECAR in the XF24 Extracellular Flux Analyzer. Additional measurements for mitochondrial respiration and glycolysis were performed after injection of four compounds (added through different ports sequentially, in each well), namely: oligomycin [(injection A: 1 μ g/mL), it blocks ATP synthase and is thus used to determine ATP turnover rates]; carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP) [(injection B: 1 μ M), it uncouples mitochondria and stimulates maximal respiration and glycolysis]; 2-deoxyglucose (2-DG) [(injection C: 10 mM), it inhibits hexokinase-the first enzyme in the glycolytic pathway]; and antimycin A [(injection D: 3 μ M), it inhibits electron transport chain and is therefore used to measure non-mitochondrial respiration]. Real-time OCR and ECAR were recorded during specific programmed time periods (3 readings each) as the average numbers between the injections of inhibitors mentioned above. In general, baseline OCR was calculated as respiration before injection of any of these compounds minus OCR after antimycin A injection, and mitochondrial respiratory reserve capacity was calculated using FCCP minus the basal OCR [27]. The final data calculations were performed after the readings were normalized with protein concentration of each well. Similarly, baseline ECAR

was calculated as the recorded acidification rate during the respiratory conditions explained earlier in this section. OCR and ECAR are expressed as picomoles per minute per microgram of protein and milli pH unit change per minute per microgram of protein, respectively. One-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA) was used to calculate statistical significance of differences between control and GSE-treated samples. *P* values of ≤ 0.05 were considered significant.

RESULTS

Identification of Protein Targets of GSE Binding by DARTS Technique

To identify potential protein targets of GSE we utilized DARTS (Fig. 1) [7-9]; GSE treatment was done at 60-100 $\mu\text{g/ml}$ doses for 3 h in a panel of human CRC cell lines; these cell lines were chosen based on phenotypic and genetic variations, so as to cover different clinical stages of CRC, viz., SW480 (stage II CRC), SW620 (stage III CRC) and HCT116 (stage IV CRC) cells [10]. The selection of doses was based on our previous study documenting the differential efficacy of GSE in different human CRC cell lines [10]. After GSE treatment and subsequent Pronase mediated proteolytic digestion of cell lysates (Fig. 1A), the remaining proteins were separated and stained via SDS-PAGE with Coomassie blue; this experiment revealed multiple enriched protein bands in the GSE group as a result of decreased susceptibility to proteolysis due to GSE-protein binding [7-9]. For example, in SW480 CRC cells, GSE treatment (100 $\mu\text{g/ml}$) resulted in enrichment of 3 main peptide bands; enriched bands occurred around 75 kDa, 53 kDa, and 52 kDa molecular weights (Fig. 2A). The DARTS technique in SW620 CRC cells revealed that GSE treatment (60 $\mu\text{g/ml}$) resulted in enrichment of 6 main peptide bands; enriched bands occurred around the molecular weights of 75 kDa, 74 kDa, 53 kDa, 52 kDa, 40 kDa, and 20 kDa (Fig. 2B). DARTS protocol in HCT116 human CRC cells [GSE treatment (60 $\mu\text{g/ml}$)] resulted in enrichment of similar peptide bands as in SW620 cells; however, the enrichment of 20 kDa band was absent (Fig. 2C). Mass spectroscopy analysis of these selective bands revealed the presence of complex mixture of peptides in these bands; a representative LC/MS chromatogram profile is shown in Supplementary Fig. 1.

To further interpret LC/MS information, peptide mass fingerprints were imported into the MASCOT search engine; via this database, statistically, the most probable proteins that were targeted by GSE treatment were identified (Table 1A & B). In SW480 CRC cells, GSE treatment potentially up regulated the expression of GRP78, PDIA3, HSP7c, DHE3 and K2C1; in SW620 CRC cells, GSE treatment enriched peptide bands specific for GRP78, PDIA3, HSP7c, NUCL, DHE3 and GRP75 (Table 1A & B); and in HCT116 human CRC cells, GSE treatment enriched specific peptide mass fingerprints for GRP78, PDIA3, GRP75, K2C1 and ALDOA (Table 1A & B). The sequence coverage of Maps for individual proteins is shown in Supplementary Fig. 2.

MASCOT results also revealed the number of peptides identified and further calculated the sequence coverage for the identified peptide mass fingerprints. As an example, examining the sequence coverage for GRP78 in these cell lines revealed a high percent of sequence coverage; specifically, the GRP78 protein peptide sequence for these cell lines was: SW480~49%; SW620 ~31% and HCT116 ~40% (Table 1B). Examining the identified

proteins, which could be possible direct targets of GSE, revealed a potential global protein modification following GSE treatment. Furthermore, examining the spectral output by LC/MS and the MASCOT data revealed that GSE exposure resulted in numerous oxidative protein modifications, specifically on methionine amino acids residues (Table 1A & B). These oxidative methionine residue modifications occurred in the following identified proteins: GRP78, PDIA3, HSP7c, DHE3, GRP75, K2C1 and ALDOA (Table 1B).

Glucose related protein-78 (GRP78), belongs to the HSP family of molecular chaperones and is required for ER integrity and stress induced autophagy [28]. Protein disulfide-isomerase A3 (PDIA3) is also localized to the ER and involved in protein folding; additionally, nucleolin (NUCL) is involved with ribosome maturation and assembly [29, 30]. Glutamate dehydrogenase 1 (DHE3) is a mitochondrial matrix enzyme; the glucose related protein-75 (GRP75) protects against oxidative stress and localized to the mitochondria [31, 32]. Additionally, GSE may interact with HSP7c, which acts as a transcriptional repressor; this protein is further activated upon cellular stress. Keratin type II cytoskeletal 1 (K2C1) is involved in kinase regulation and responses to oxidative stress stimuli [33]. Finally, fructose- biphosphate aldolase (ALDOA), a key enzyme involved in glycolysis, is a potential target of GSE [34]. The above potentially identified proteins that were modified by GSE treatment and their cellular localization are summarized in Fig. 3.

Validation of GSE Protein Targets in Human CRC Cells

The DARTS technique, combined with LC-MS analysis and MASCOT, identified potential protein targets of the GSE components. A number of aforementioned proteins as summarized in Fig. 3, are involved in protein folding and ER integrity. To validate the potential effect of GSE treatment on ER integrity, we investigated GSE effect on ER stress and ER stress pathways in these human CRC cell lines. Immunofluorescence studies utilizing ER-ID Red dye indicated significant ER stress caused by GSE as visualized by increase in red ER staining in GSE treated CRC cells; representative photomicrographs of GSE effect in HCT 116 cells are shown in Fig. 4A. Western blotting results indicated that GSE treatment (20-50 µg/ml) of SW480, SW620 and HCT116 CRC cells resulted in dramatic down-regulation of GRP78 (Fig. 4B). Calnexin protein levels were unchanged after 12h of GSE treatment in two cell lines; however, IRE1α protein levels decreased in two cell lines (Fig. 4B). Additionally, GSE treatment of SW480 and SW620 CRC cells resulted in a down-regulation of ATF6α; however, no ATF6α protein alteration was observed in HCT116 cells (Fig. 4B). Furthermore, eIF2α protein levels were increased in SW480 CRC cells; in contrast, eIF2α levels were decreased in SW620 and HCT116 CRC cells (Fig. 4B). We also examined DHE3 levels in SW480 and SW620 CRC cells; results indicated an increased expression of DHE3 in SW480 CRC cells; however, no change in protein levels was observed in SW620 cells (Fig. 4C). Examining the potential extracellular matrix as targets of GSE indicated no direct effect on protein levels of K2C1 and a known binding partner Integrin β1 in HCT116 CRC cells (Fig. 4C).

Effect of GSE on Activated PI3K-Akt-mTOR Pathway in Human CRC Cells

One of the hallmarks of cancer cells is apoptosis resistance. Whereas our results shown in Figs. 3 and 4 and Table 1 clearly showed that GSE targets ER stress and associated

pathways in human CRC cell lines, which would be useful to overcome apoptosis resistance in these cells, there are other cell survival and mitogenic signaling pathways which are also impaired in cancer cells including CRC that, when constitutively activated, results in apoptosis resistance. For example, growth factor signaling [(such as insulin-like growth factor (IGF-1) induced signals)] governed by the receptor tyrosine kinases and mediated via the PI3K/Akt and the MAPK pathway is often deregulated and constitutively activated in cancer cells [35-37]. In CRC, IGF-1 is a potent mitogen that regulates proliferation and survival of CRC cells *via* both Akt- and MAPK- mediated signaling [26, 36, 37], which suggests that agents are needed to also impair such signaling to achieve better success in overcoming apoptosis resistance in CRC cells. Accordingly, we also performed additional studies to assess whether, in addition to targeting ER stress and associated pathways, GSE also inhibits survival and mitogenic signaling in human CRC cells. Indeed, GSE inhibited both constitutive and IGF-1-induced Akt activation by inhibiting the activation of IRS-1 of the IGF-1R β pathway (Fig 5A & 5B). Whereas above results suggested that GSE would also inhibit ERK1/2 activation, it resulted in an increase in ERK1/2 phosphorylation, which was dose dependent; this phenomenon has been also observed earlier with GSE [16]. Further mechanistic studies revealed that GSE strongly inhibits the activation of IGF-1-induced Akt-mTOR pathway. Specifically, GSE significantly decreased IGF-1 induced phosphorylation of mTOR and the downstream target P70S6K. Furthermore, GSE also decreased the phosphorylation of eukaryotic initiation factor 4E binding protein-1 (4E-BP1), which controls protein translation, [38] at various sites in both SW480 and HCT116 cell lines studied (Fig 5A & 5B). GSE also decreased the phosphorylation of various other translation initiation factors such as EIF-4G and EIF-4B. The decrease in phosphorylation of these proteins in Akt-mTOR pathway was also associated with an overall decrease in their total proteins by GSE (Fig 5A & 5B). The increase in phosphorylation of eukaryotic translation elongation factor 2 (EEF2) in SW480 cells by GSE indicated that GSE also could inhibit translation elongation; however this protein could not be visualized in HCT116 cells (Fig 5A & 5B). Overall, these results indicated that GSE interferes in cellular protein synthesis in CRC cells by its ability to differentially modulate both the initiation and elongation steps of translation.

Effect of GSE on Bioenergetics of Human CRC Cells

It is well known that growth factor driven PI3K-Akt pathway also regulates tumor cell metabolism where activated PI3K/Akt leads to increased uptake of glucose and glycolysis in most of the human cancer cells, including CRC. Based on our data showing that GSE significantly inhibits both constitutive and IGF-1-induced PI3K/Akt pathway activation, we next analyzed the bioenergetic/metabolic alterations in human CRC cells SW480 and HCT116 after GSE treatment. The Seahorse XF24 extracellular flux analyzer was employed (Figure 6A & B) to measure oxygen consumption rate-OCR (determinant of oxidative phosphorylation) and extracellular acidification rate-ECAR (determinant of glycolysis). As shown in Figure 6C & D (*left panel*) treatment with GSE for 9h significantly decreased the baseline OCR in CRC cells ($P = 0.05$). To assess the effect on ATP turnover in CRC cells, OCR was analyzed in response to oligomycin addition in both control and GSE treated cells. Results showed that there was a significant decrease in ATP turnover in GSE treated SW480 cells compared to controls (Fig. 6C, *right panel*); however, in HCT116 cells while a decrease

in ATP turnover was observed (Fig. 6D, *right panel*), it was not statistically significant. We also observed that GSE causes a reduction in proton leak in both SW480 (Fig. 6C, *left panel*) and HCT116 cells (Fig. 6D, *left panel*). Similarly, mitochondrial reserve respiratory capacity was measured before and after adding FCCP, followed by the addition of antimycin A. Overall it was observed, that FCCP addition causes an increase in OCR, while the addition of antimycin A causes a sharp decrease in OCR under all treatment conditions (both in the absence and presence of GSE). Importantly, while there was a significant decrease in mitochondrial reserve respiratory capacity in GSE treated HCT116 cells compared to controls, the same effect was not seen in SW480 cells (Fig 6C & D, *right panel*). We also determined the glycolytic rate (indicated by ECAR) in both control and GSE-treated CRC cells. It was seen that there was a significant decrease in ECAR in both CRC cell lines after GSE treatment (Fig 6C & D, *bottom panel*), which indicated towards a significant decrease in glycolysis after GSE exposure.

DISCUSSION

Natural products have been used exclusively for thousands of years; these natural products were the ultimate source of small molecule drugs exhibiting numerous beneficial effects that have been used since 2737 B.C. [5, 7-9, 12]. There are millions of species that contain compounds with valuable pharmacological properties, including chemopreventive and anti-cancer potential; only in the last half a century has the scientific community begun to tap into the vast source of scientific knowledge within the environment [5, 7, 9, 12]. Even though, these natural agents are widely consumed as dietary supplements, the specific protein targets of these agents are not known and their use in clinical applications is marred by limited scientific evidences for their beneficial effects [5, 7, 9, 12]. Taken the above information, and the fact that the natural agent, GSE, has shown considerable efficacy against CRC in pre-clinical studies [4, 10-12, 14-16, 20, 21], the results of the current study using DART technology elucidated the upstream stimulus responsible for the chemopreventive and anti-cancer efficacy of GSE in CRC model. These results indicated the initial mechanism of action of GSE, which was responsible for its anti-cancer efficacy; specifically how GSE targets ER stress response proteins, resulting in an overall down regulation of proteins involved in translation. Specifically, we observed oxidative protein modification, specifically on methionine amino acids residues, and an altered ER stress response protein expression, in human CRC cells, as a result of GSE treatment.

The ER is responsible essentially for cellular protein folding and secretion, biosynthesis of lipids, and calcium homeostasis [39]. The ER is also a major cell organelle for sensing oxygen and nutrients; the stresses that disrupt ER function, from the surrounding cellular microenvironment, result in the accumulation of unfolded proteins [39]. The ER stress response can be triggered following imbalances in cellular homeostasis; this cellular response can adopt a pro-survival effort or a pro-apoptotic effort [28]. GRP78/BiP is a major player in the ER stress response system; notably, GRP78 is reported to be frequently up regulated in cancer cells [28], and importantly, it is an effective specific target that we found is modified by GSE. Oxidative modification of GRP78 was detected at 3h of GSE treatment in two human CRC cell lines; this protein modification further resulted in down regulation of GRP78 protein levels after 12 h of GSE treatment, which might be a selective GSE effect

in inducing apoptotic death in CRC cell lines but not normal colon epithelial cells as reported by us recently [10]. Furthermore, GSE treatment also resulted in the down regulation of downstream targets of GRP78, namely IRE1 α , ATF6 α and eIF2 α . IRE1 α is triggered, by GRP78, in response to the accumulation of unfolded proteins within the ER lumen [40]. Regarding ATF6 α , its levels were decreased in response to GSE treatment in SW480 and SW620 cells; ATF6 α senses ER protein mis-folding stress and is further involved in protein secretion and degradation within the golgi apparatus [41]. Furthermore, GSE treatment in SW480 cells resulted in up regulation of eIF2 α ; while in the other two cell lines HCT116 and SW620, GSE treatment resulted in a down regulation of eIF2 α . The eIF2 α protein is another sensor that is typically up regulated due to ER stress; this eIF2 α pathway is also activated via ERK signaling [42]. In CRC cells, GSE treatment has been shown to cause up regulation of ERK phosphorylation and activation in a cyclic pattern, which is dose- and cell line-dependent [16]. Together, these results coincide with the altered eIF2 α expression pattern in these CRC cell lines by GSE treatment; importantly, the ability of GSE to also down regulate eIF2 α indicates that GSE induces oxidative stress, leading to the accumulation of un-translated and unfolded proteins. Furthermore, GSE's ability to decrease pan-signaling protein GRP78, resulting in down regulation of downstream protein pathways namely IRE1 α , ATF6 α and eIF2 α , possibly also resulted in the inhibition of the unfolded protein response; this inhibition results in damage to the ER membrane causing a loss of membrane potential as observed by us employing immunofluorescence assay utilizing ER-ID Red dye. Indeed, GSE has been previously shown to decrease mitochondrial potential in these CRC cells as well as in prostate cancer cells [10, 43]. In addition, the loss of ER membrane potential could also alter intra-cellular Ca²⁺ levels; altered Ca²⁺ levels have been shown to further result in IRE1 α protein alteration. The intra-cellular Ca²⁺ levels can also result in mitochondrial membrane permeability further leading to the activation of caspase-8 and caspase-9; recent mechanistic studies investigating GSE efficacy in these CRC cell lines have revealed the activation of both caspase-8 and -9 as a result of GSE treatment, leading to cancer cell apoptotic death [10].

As has been reported earlier, Akt plays a significant role in cellular stress conditions, which impairs ER function [44]. Previous studies also suggest that, BH- containing proteins, which regulate ER stress-mediated apoptosis activation, are similar to those involved in mitochondrial apoptotic pathway. It is also suggested that mammalian TOR (mTOR)'s signaling, as a sensor for amino acids and ATP, is an integrator of signals transduced by growth factors that eventually are involved in both mitochondrial and ER apoptotic pathways [45, 46]. mTOR plays a central role in the regulation of essential cellular processes including metabolism, proliferation, and differentiation [46]. mTOR's downstream effect is mediated by p70S6K and eukaryotic initiation factor 4E binding protein-1 (4E-BP1) that controls protein translation [38, 47]. To determine whether GSE could induce interference in cellular protein translation *via* modulation of mTOR pathway, we carried out studies in presence of IGF-1 to understand the specific effect of GSE on activated Akt/mTOR pathway. Indeed, GSE treatment of CRC cells inhibited both the constitutively active and IGF-1-induced activation of PI3K/Akt/mTOR pathway. Furthermore, it is also known that growth factor driven PI3K-Akt pathway also regulates tumor cell metabolism [48] where activated PI3K/Akt leads to enhanced uptake of glucose

and increased glycolysis in most of the human cancer cells, including CRC [49]. Given that GSE caused a significant inhibition of PI3K/Akt pathway, using XF24 Extracellular Flux Analyzer, we also determined GSE effect on cellular bioenergetics in CRC cells. Since cancer cells have an aberrant cellular metabolism and are more reliant on glycolysis for rapid proliferation and metastasis, we investigated the effect of GSE on both oxidative phosphorylation (represented by OCR) and glycolysis (represented by ECAR). Indeed, consistent with an inhibition of PI3K/Akt activation, GSE showed a significant decrease in both OCR and ECAR in CRC cells. The deficient glucose metabolism activity has been suggested to contribute towards insufficient ATP production during oxidative phosphorylation indicating the potential of GSE to modulate bioenergetics of CRC cells. Collectively, these results suggest that targeting both glycolysis and oxidative phosphorylation by GSE may be a promising chemopreventive and/or chemotherapeutic strategy against CRC. However, more molecular and genetic approaches are required to understand the exact molecular mechanism behind GSE-mediated changes in bioenergetics of CRC cells.

Of central importance to metabolic pathways are the amino acids glutamine and glutamate; the key enzymes involved in their synthesis are localized almost exclusively to the mitochondria [50-52]; specifically, glutamate dehydrogenase (DHE3) is localized to the inner surface of the mitochondria membrane and is involved in glutamate synthesis [50]. Alteration of glutamine metabolism occurs in many forms of cancer; this is due to the fact that glutamine is a precursor to purine and pyrimidine synthesis and it also is the major substrate for tumor energy metabolism [50-52]. Additional investigation of GSE potential protein targets revealed that in SW480 cells, GSE protein oxidation resulted in up regulation of DHE3 expression; however, this effect was not observed in SW620 and HCT116 CRC cell lines. Further enzyme activity studies would be ideal to identify and validate DHE3 activity post GSE treatment in these CRC cell lines. Similarly, the cellular cytoskeletons are key players in organizing cytoplasmic organelles, defining cell polarity, and generating pushing and contractile forces [53]. To further investigate GSE ability to alter cellular structural proteins, we examined the protein expression of K2C1 and its known binding partner Integrin β 1; results revealed no alteration in K2C1 or Integrin β 1 expression levels in response to GSE treatment. However, these findings do not rule out the possible role that oxidation of K2C1 could result in such as alteration of kinase activity, activation of an oxidative stress response and further alteration in cellular morphology. Further examination of PKC and SRC kinase activity may reveal the role of K2C1 oxidation in response to GSE treatment.

Overall, this study indicates that DARTS is a useful affinity technique that allows researchers to identify potential protein targets of small molecules within complex protein mixtures. Specifically, DARTS identified eight overall proteins that could be potential targets of GSE treatment in human CRC cells; these potential targets are: GRP78, PDIA3, HSP7c, NUCL, DHE3, GRP75, K2C1, and ALDOA. Furthermore, the LC/MS data collected in this study warrants further investigation into the other potential protein targets of GSE; additional proteins were identified via MASCOT but we did not focus on them in the present study due to low sequence coverage. In summary, identifying the potential targets of GSE treatment involved in its anti-cancer and chemopreventive efficacy against

CRC, allows the further development of this natural supplement in the clinical setting. This information, combined with the vast pre-clinical GSE efficacy studies, would further solidify GSE as a safe, effective, multi-targeted anti-cancer and chemopreventive agent for CRC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

2-DG	2-deoxyglucose
4E-BP1	Eukaryotic initiation factor 4E binding protein-1
ATF6α	Endoplasmic reticulum stress-activated transcription factor
ATP	Adenosine triphosphate
CRC	Colorectal cancer
DARTS	Drug affinity responsive target stability
DMSO	Dimethyl Sulfoxide
ECAR	Extracellular acidification rate
EEF2	Eukaryotic translation elongation factor 2
eIF2α	Eukaryotic initiation factors2 α
EIF-4B	Eukaryotic initiation factor-4B
EIF-4G	Eukaryotic initiation factor-4A
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated protein kinase
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide 4-trifluoromethoxyphenylhy-drazone
GRP78	78 kDa glucose-regulated protein
GSE	Grape seed extract
HSP7c	Heat shock cognate 71 kDa protein
IGF-1	Insulin-like growth factor-1
IRE1α	Inositol-requiring enzyme 1 α
IRS	Insulin receptor substrate
K2C1	Keratin type II cytoskeletal 1
LC/MS	Liquid chromatography–mass spectrometry
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
NUCL	Nucleolin
OCR	Oxygen consumption rate
PDIA3	Protein disulfide isomerase family A, member 3
PI3K	Phosphoinositide 3-kinase

Fig. (1). Scheme of events to depict treatment of human CRC cells with GSE resulting in binding of GSE components to target proteins. The proteins were isolated and subjected to proteolysis by DARTS technique, further separated and finally identified via LC/MS.

Fig. (2).

Identification of protein targets of GSE binding in human CRC cells by DARTS technique. Human CRC cells were treated with GSE [(A) SW480, and (B) SW620 cells (100 $\mu\text{g/ml}$ GSE), (C) and HCT116 cells (60 $\mu\text{g/ml}$ GSE)] resulting in binding of GSE components to target proteins; these proteins were isolated and subjected to proteolysis *via* the DARTS technique. The proteolytic left-over products of the control and GSE treated samples were separated via SDS-PAGE, followed by Commassie staining. Different lanes indicate varying ratio of Pronase vs. cell lysates protein concentration [1:25, 1:50, and 1:100] used to digest the protein samples. UD, undigested sample.

Fig. (3).

Summary of potential protein targets of GSE in human CRC cells. Illustration depicts the cellular localization of proteins that were identified by DARTS technique as potential proteins targets (binding) of GSE components (leading to their subsequent modification) in human CRC cells; the selected proteins are the ones that presented the greatest sequence coverage in our MASCOT analysis. Multiple proteins were potentially identified via LC/MS analysis coupled with MASCOT protein identification. The protein targets are as follows: GRP78; PDIA3; HSP7c; NUCL; DHE3; GRP75; K2C1 and ALDOA. R-ER, rough endoplasmic reticulum.

Fig. (4).

Validation of GSE caused ER stress and DARTS identified protein targets in human CRC cells. **(A)** Effect of GSE on ER stress generation in HCT116 cells as visualized using ER-ID Red dye under confocal microscope. ER images are captured in red and blue shows DAPI stained nuclei. Magnification ($60\times \times 2.3$). **(B)** Effect of GSE treatment on the protein levels of molecules involved in ER stress viz., GRP78, IRE1α, ATF6α, eIF2α, and calnexin in human CRC cells. **(C)** Effect of GSE treatment on mitochondrial and cytoskeleton proteins viz., DHE3, K2C1, and Integrin β1 in human CRC cells. Briefly, SW480, SW620 and HCT116 were treated with different concentrations of GSE (20-50 µg/mL) or DMSO alone (control) for 12 hours. At the end of treatment, both adherent and non-adherent cells were harvested and cell lysates were prepared in non denaturing lysis buffer and immunoblotting was performed as detailed in Materials and Methods section.

Fig. (5).

Effect of GSE on constitutively activated and IGF-1 activated PI3K/Akt/mTOR and ERK1/2 pathway in human CRC cells. Serum starved CRC cells were treated with GSE for 6h followed by IGF-1 treatment for 15 min. Cell lysates were subjected to immunoblotting, and all experimental procedures were performed as detailed in 'Materials and Methods' section. Some immunoblots were also stripped and then used again (reprobed) with different antibodies, such as loading control (β -actin).

Fig. (6).

Effect of GSE on bioenergetics of human CRC cells. Modulation in bioenergetics of human CRC cells after GSE treatment (9h) was measured using XF24 extracellular flux analyzer as detailed in 'Materials and methods'. **(A-B)** Graphical representation of overall OCR profiling in response to different inhibitors in SW480 and HCT116 cells. Black arrows mark the sequential and programmed addition of Oligomycin, FCCP, 2-DG, and Antimycin A in wells, through different ports **(C-D)** Changes in basal OCR, ATP turnover, proton leak, reserve respiration capacity and basal ECAR in **(C)** SW480, and **(D)** HCT116 cells after GSE exposure. The representative data are expressed as mean $\pm$ SEM normalized with respective protein concentrations, and each experiment was performed in quadruplicate at least thrice. The significant differences between groups were calculated by one way ANOVA. *, $P < 0.001$; \$, $P < 0.05$.

Table 1
A. MASCOT protein identification of GSE targets by DARTS technique

Proteins enriched with GSE	SW480	SW620	HCT116
GRP78	X	X	X
PDIA3	X	X	X
HSP7c	X	X	
NUCL		X	
DHE3	X	X	
GRP75		X	X
K2C1	X	X	X
ALDOA			X

B. Identification of protein modifications in response to GSE treatment				
SW480				
Protein Name	No of Peptides Identified	Sequence Coverage (%)	Amino Acid Residue Modified	Modification
GRP78	31	48.80	Methionine	Oxidation
PDIA3	22	41.60	Methionine	Oxidation
HSP7c	10	17.60	Methionine	Oxidation
DHE3	22	43.00	Methionine	Oxidation
K2C1	20	29.80	Methionine	Oxidation
SW620				
Protein Name	No of Peptides Identified	Sequence Coverage (%)	Amino Acid Residue Modified	Modification
GRP78	19	31.30	Methionine	Oxidation
PDIA3	20	35.80	Methionine	Oxidation
HSP7c	18	29.40	Methionine	Oxidation
DHE3	17	30.60	Methionine	Oxidation
GRP75	31	40.10	Methionine	Oxidation
K2C1	20	26.20	Methionine	Oxidation
HCT116				
Protein Name	No of Peptides Identified	Sequence Coverage (%)	Amino Acid Residue Modified	Modification
GRP78	27	40.20	Methionine	Oxidation
PDIA3	26	42.60	Methionine	Oxidation
GRP75	35	40.50	Methionine	Oxidation
K2C1	26	32.90	Methionine	Oxidation
ALDOA	22	50.80	Methionine	Oxidation