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Isothiocyanates from Broccolini seeds induce apoptosis in human colon cancer cells: proteomic and bioinformatic analyses

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Received October 7, 2010, accepted November 11, 2010

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Pharmazie 66: 382–390 (2011) *doi: 10.1691/ph.2011.0810*

Isothiocyanates (ITCs) have been shown to possess antitumor activity in colon cancer, however, the detailed mechanism is still unclear. The objective of this study was to investigate apoptosis-inducing activity of ITCs from Broccolini seeds and proteomic changes in SW480 cells, and to identify the molecular pathways responsible for the anticancer action of ITCs. We found that ITCs induces SW480 cells apoptosis in a dose-dependent manner by using MTT assay, phase contrast microscope and flow cytometry, and the IC50 was calculated to be 77.72 μg/ml, superior to the chemotherapeutical drug 5-flurouracil. Subsequently, 15 altered proteins in ITCs treated SW480 cells were identified. Further bioinformatics analysis predicted the potential pathways for ITCs to induce apoptosis of SW480 cells. In conclusion, this is the first report to investigate anticancer activity of ITCs from Broccolini seeds and its mechanism of action by proteomics analysis. Our observations provide potential therapeutic targets for colon cancer inhibitor intervention and implicate the development of novel anti-cancer therapeutic strategies.

1. Introduction

Epidemiological studies suggest that the dietary consumption of isothiocyanates (ITCs), compounds found primarily in cruciferous vegetables (such as broccoli, Brussels sprouts, cauliflower, and cabbage), are associated with a decreased cancer risk. Various ITCs, including allyl-ITC (AITC), benzyl-ITC (BITC), phenethyl-ITC (PEITC), sulforaphane (SFN), erucin (ERN) and iberin (IBN), have been shown in laboratory studies to possess antitumor activity in several forms of cancer (Mas et al. 2007; Clark et al. 2008). Mechanistically, ITCs are capable of inhibiting both the formation and development of a cancer cell through multiple pathways; i.e., the inhibition of carcinogen-activating cytochrome P450 mono-oxygenases, induction of carcinogen-detoxifying phase 2 enzymes, induction of apoptosis, and inhibition of cell cycle progression (Cheung and Kong 2010; Nakamura 2009).

Colon cancer represents one the most important causes of premature death in developed countries. The successful treatment for this cancer is still limited, significant attention is currently being paid to preventive programs which may interfere with the process of colon carcinogenesis at all its stages. ITCs has proved to be an effective chemoprotective agent in colon cancer. For example, SFN acts to induce multivariate cascades including DNA-damage response pathway in human colon cancer cells SW620 (Rudolf et al. 2009); PEITC was also shown to induce apoptosis and cell arrest through MAPK pathway in colon cancer cells (Cheung et al. 2008); BITC has been shown to have the antimetastatic effects in human colon cancer HT29 cells (Lai et al. 2010). Despite the reported cytotoxicity and apoptosis-inducing properties of these ITCs in colon cancer cells, the details concerning individual mechanisms and signaling pathways underlying the apoptosis induction remain unclear.

Advances in the use of proteomic technologies provide a robust approach to study multiple signaling pathways simultaneously. The altered proteins identified by a proteomic approach can be further characterized as potential drug targets (Ge et al. 2009). Broccolini, known in Europe as asparation and in the United States as baby broccoli, is a hybrid between broccoli and Gai Lan, also known as Chinese broccoli and Chinese kale. We assume that the ITCs from Broccolini also have antitumor activity. The purpose of this study was to investigate the potency of apoptosis induction in human colon cancer cells SW480 by the ITCs in extracts from Broccolini seeds, and to elucidate their potential anticancer mechanisms using proteomic technology (i.e. the combinantion of 2-dimensional gel electrophoresis (2-DE) and MALDI–TOF–TOF mass spectrometry). Based on this, we expect to discover new potential therapeutic targets for dietary intervention of colon cancer and provide direct implications for the development of novel anti-cancer therapeutic strategies.

2. Investigations and results

2.1. ITCs induces apoptosis in SW480 cells

The MTT measurement results showed that ITCs (10∼120 μg/ml) can exert a remarkable inhibitory effect on the growth of SW480 cells. Treating the cells with ITCs and anti-cancer drug 5-FU (10∼120 μg/ml) resulted in a dose-dependent inhibition of cellular proliferation. The IC50

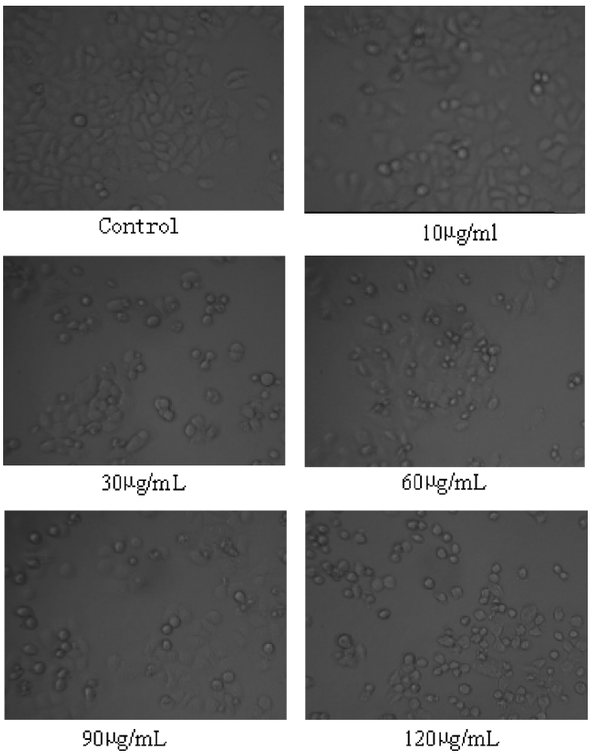


Fig. 1: ITCs induced morphologic changes in SW480 cells observed by phase contrast microscopy

values of ITCs and 5-FU in SW480 cells were estimated to be 77.72 μg/ml and 186.34 μg/m, respectively. This means that ITCs exhibit a stronger anti-proliferation ability in SW480 cells than the widely used chemotherapeutical drug 5-FU.

In morphology analysis, phase contrast microscopy was used and the results showed that the attached cell grew well for the negative control (Fig. 1). However, compared with the negative control group, the ITCs (10∼120 μg/ml) treatment groups were significantly different. In low-dose groups (10∼90 μg/ml), the round detached cells started to appear and the cell density was gradually reduced. In the high-dose group (120 μg/ml), the morphology structure of SW480 cells become irregular, which exhibited characteristics of apoptosis such as cell membrane shrinkage, condensation and fragmentation of nuclear chromatin as well as formation of apoptotic bodies (Fig. 1). Together, these results suggested that ITCs could induce apoptosis and necrotic death of SW480 cells.

To further investigate the underlying mechanism of the anti-proliferative activity, we performed flow cytometric analysis. Based on the characteristic of apoptotic cells, unaffected cells, early apoptotic cells, and late apoptotic cells were distinguished by double staining of cells with Annexin V-FITC and PI. In the ITCs-treated SW480 cells for 24 h with 10, 30, 60, 90, 120 μg/mL, the percentage of apoptotic tumor cells were found to be 1.94%, 42.60%, 49.08%, 52.06% and 76.43%, respectively (Fig. 2). Thus, flow cytometric analysis also shows that ITCs reduces proliferation of SW480 cells by inducing apoptosis and necrotic death in a dose-dependent manner.

2.2. 2-DE gel comparison

In order to compare the protein expression profiles of SW480 cells with and without ITCs treatment, samples of 450 μg protein were directly extracted with the urea lysis solution and separated by 2D-PAGE, the results are shown in Fig. 3A and B. To obtain statistically significant results, each protein sample was run in triplicate. The proteomic maps of ITCs-treated cells and control were compared using PDQuest program to identify the protein spot variations. As a result, 107 protein spots were detected, which are significantly differentially expressed protein spots ($p < 0.05$) with 2-fold or more increased or decreased intensity as observed in all three replicate gels, after ITCs treatment (Fig. 3C). Of them 75 protein spots were upregulated, and 32 downregulated.

2.3. Protein identification

Of the 107 differentially expressed proteins after ITCs treatment, top 15 most differentially expressed protein spots (Fig. 3D and E) were excised manually, digested and finally identified by peptide mass fingerprinting using MALDI-TOF-MS/MS followed by database search. Consequently, 15 protein spots were identified successfully, of which five were upregulated and ten were downregulated. The Table shows their detailed information including protein MW/p*I*, IPI database accession numbers, Mascot Score, numbers of peptides matched, and coverage (Percentage of predicted protein sequence covered by matched peptides). In ITCs-treated SW480 cells, five increased proteins were PPIA (peptidylprolyl isomerase A), ACTG1 (Actin, gamma 1) GSTP-1 (glutathione S-transferase P), HSPB1 (heat shock 27 kDa protein beta-1), and PRDX1 (peroxiredoxin-1). Ten decreased proteins include: NME1 (nucleoside diphosphate kinase A), SOD1 (superoxide dismutase 1), STMN1 (stathmin), PARK7 (Parkinson disease protein), ANXA1 (annexin A1), SEPT2 (septin 2), LDHB (lactate dehydrogenase B), PRDX3 (peroxiredoxin 3), OXCT1 (3-oxoacid CoA transferase 1), PRDX4 (peroxiredoxin 4). In particular, among the 10 decreased proteins, SOD1 and LDHB were remarkably down-regulated, undetectable in ITCs-treated samples. Based on PANTHER classification system, the identified 15 proteins can be classified into several functional categories: Cell communication (STMN1), immunity and defense (SOD1, PPIA), metabolism (for fatty acid, pyrimidine, carbohydrate or coenzyme) (ANXA1, NME1, OXCT1), antioxidation and free radical removal (PRDX1, PRDX3, PRDX4), cytokinesis (ACTG1, SEPT2), protein folding (PPIA, HSPB1), glycolysis (LDHB), cell structure and motility (ACTG1, ANXA1), detoxification (GSTP1) and stress response (HSPB1, PARK7) (Table). A representative MALDI-TOF-MS, MS/MS and peptide sequence of spot 10 (PRDX1) is shown in Fig. 4.

2.4. Protein-protein interaction network analysis for the identified proteins

To explore the underlying pathways of apoptosis in SW480 cells induced by ITCs, protein-protein interaction network analysis was performed on the 15 identified proteins. Firstly, PPI spider was employed and 13 of the 15 identified proteins, except for PRDX3 and OXCT1, can be mapped a protein-protein interaction sub-network (Fig. 5A). In this network, several interacting proteins were introduced: FPR1 (formyl peptide receptor 1, GeneID: 2357), RIPK3 receptor-interacting serine-threonine kinase 3, GeneID: 11035), 14-3-3 zeta (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, GeneID: 7534), GRB2 (growth factor receptor-bound protein 2, GeneID: 2885), HSP90AA1 (heat shock protein 90kDa alpha (cytosolic), class A member 1, GeneID: 3320), RIPK2 (receptor-interacting serine-threonine kinase 2, GeneID: 8767), APP (amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer disease), GeneID: 351), IKBKE (inhibitor of kappa light polypeptide gene

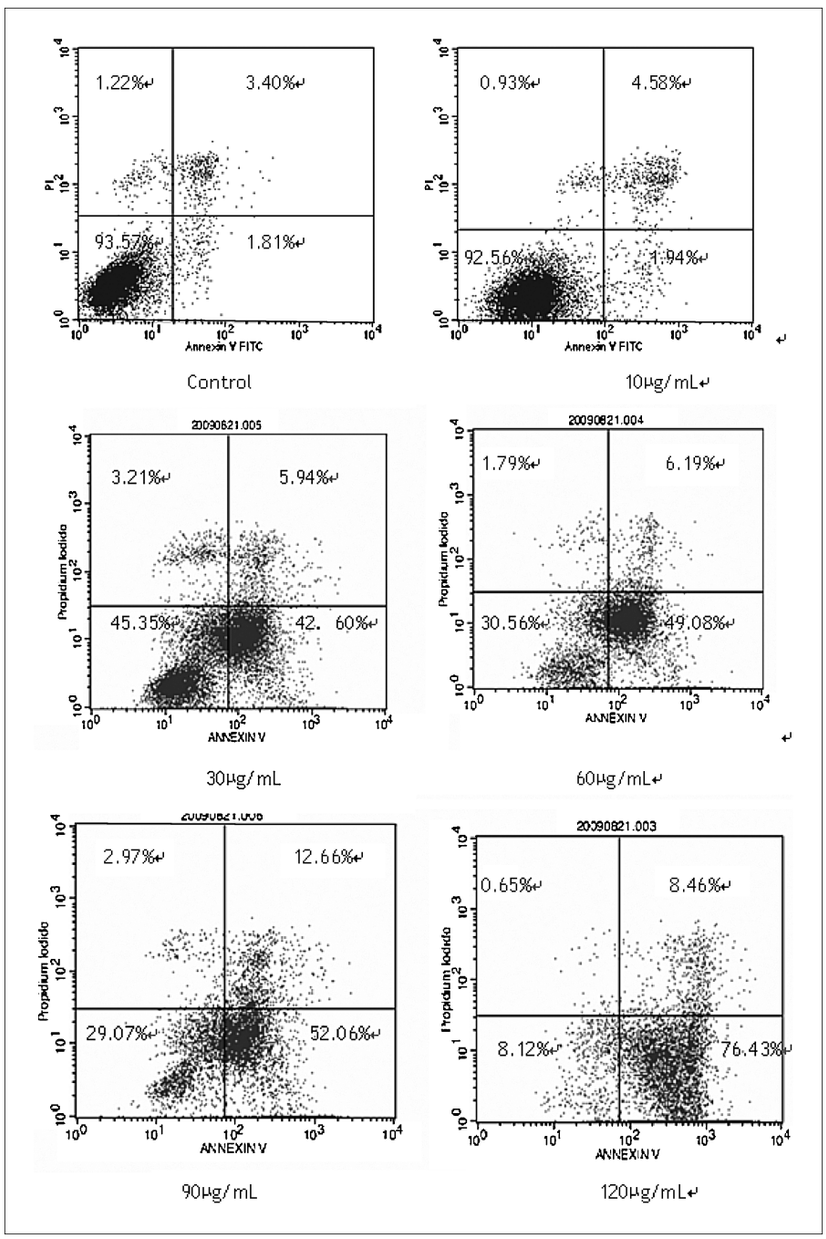


Fig. 2: ITCs-induced apoptosis analysis of SW480 cells using flow cytometry

enhancer in B-cells, kinase epsilon, GeneID: 9641), VHL (von Hippel-Lindau tumor suppressor, GeneID: 7428), SET (SET translocation (myeloid leukemia-associated), GeneID: 6418), AURKA (aurora kinase A, GeneID: 6790), TP53 (tumor protein p53 (Li-Fraumeni syndrome), GeneID: 7157), BCLl2 (B-cell CLL/lymphoma 2, GeneID: 596), DAXX (death-associated protein 6, GeneID: 1616), MAPK8 (mitogen-activated protein kinase 8, GeneID: 5599), TRAF2 (TNF receptor-associated factor 2, GeneID: 7186). Notably, among the 15 identified proteins, 5 proteins (down-regulated ANXA1 and LDHB, up-regulated PRDX1, HSPB1 and PPIA) are the direct interactors of 14-3-3 zeta. Moreover, 5 up-regulated proteins (PRDX1, HSPB1, PPIA, ACTG1 and GSTP1) are the direct interactors of GRB2. These suggest that 14-3-3 zeta and GRB2 may play a vital role in mediating ITCs-induced apoptosis in colon cancer cells.

Secondly, STRING was employed and 15 identified proteins can be mapped a protein-protein interaction sub-network (Fig. 5B). In this network, several functional partners were predicted: AR (androgen receptor), BSG (basigin precursor), TMSL1 (thymosin beta-4), MAPKAPK2 (MAP kinase-activated protein kinase 2), SEPT6 (septin-6), DSTN (destrin), CAT (catalase), SOD2 (superoxide dismutase [Mn]), SET (SET translocation (myeloid leukemia-associated)), GSTT1 (glutathione S-transferase theta-1). It can be seen from this sub-network, SOD1 and SOD2 seems become the interaction center: the identified SOD1 is directly accociated with 6 identified partners (LDHB, PRDX3, HSPB1, PRDX4, PRDX1, PARK7), the introduced SOD2 has 8 interactors among 15 identified proteins (LDHB, PRDX3, GSTP1, HSPB1, PRDX4, PRDX1, SOD1, PARK7). These hint that SOD1 and SOD2 could be very important mediators in ITCs-induced apoptosis in colon cancer cells. Furthermore, i2d database was used to search protein association network for the identified proteins. All the 15 identified proteins were mapped to a complex protein network (Fig. 5C):

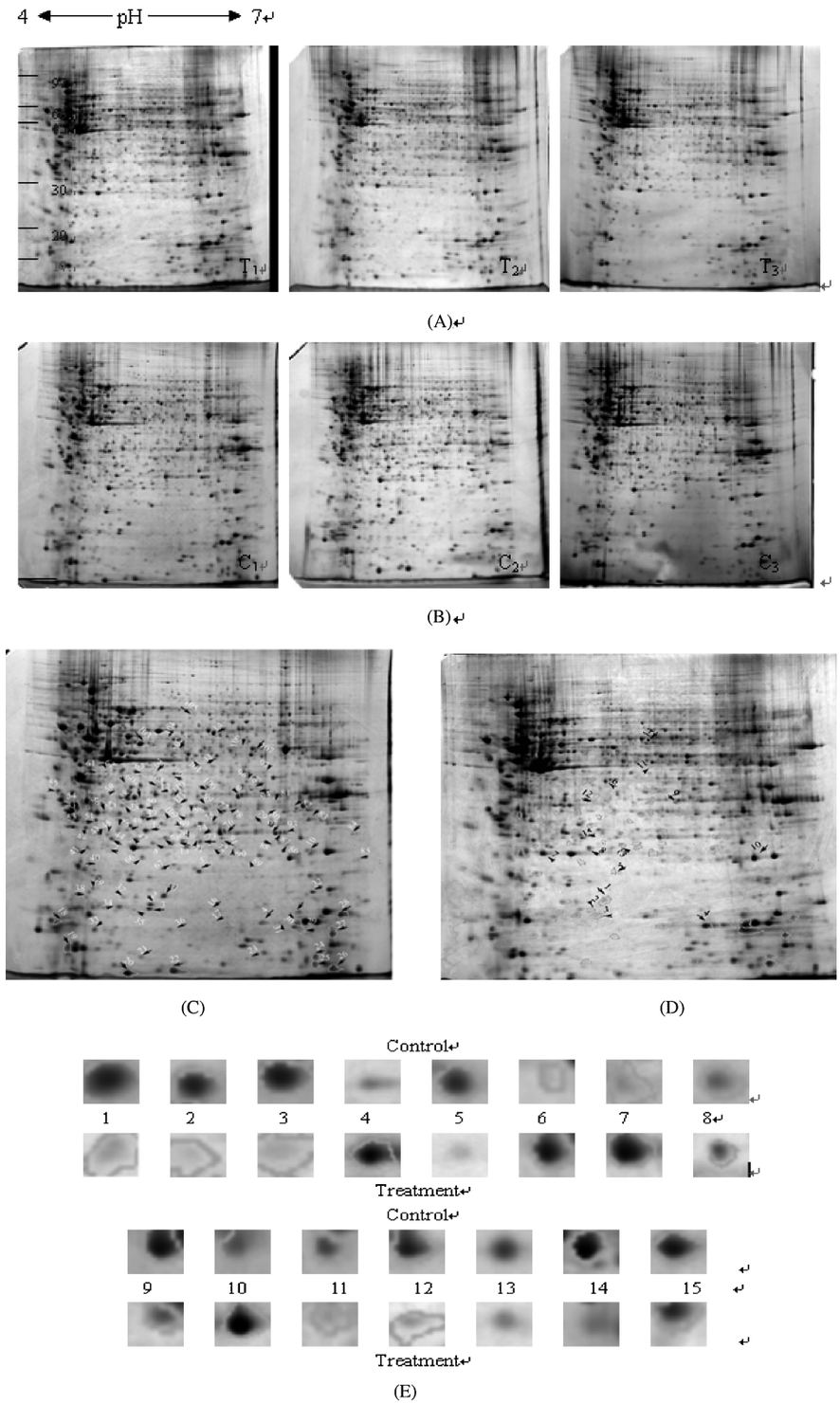


Fig. 3: 2D gel proteome map from SW480 cells with molecular weight and pH indicated on the left and head of the 2-DE gels, respectively. The labeled cell proteins were mixed and subjected to 24 cm pH 4–7 IEF electrophoresis followed by SDS-PAGE isolation. The gels were stained with Coomassie blue G-250 and the synthetic gel images were generated using PDQUEST program. (A). Image of proteins from SW480 cells with ITCs treatment. (B). Image of proteins from SW480 cells without ITCs treatment. (C). Differentially expressed protein spots. (D). Fifteen of the interest proteins are identified with the help of MALDI-TOF MS and MS/MS. (E). Fifteen of the interest protein spots are enlarged with curve lines

for example, 105 interactions for SOD1, 74 interactions for ACTG1, 41 interactions for HSPB1, 24 interactions for SEP2, 18 interactions for ANXA1, 16 for PPIA, and 13 interactions for PARK7.

3. Discussion

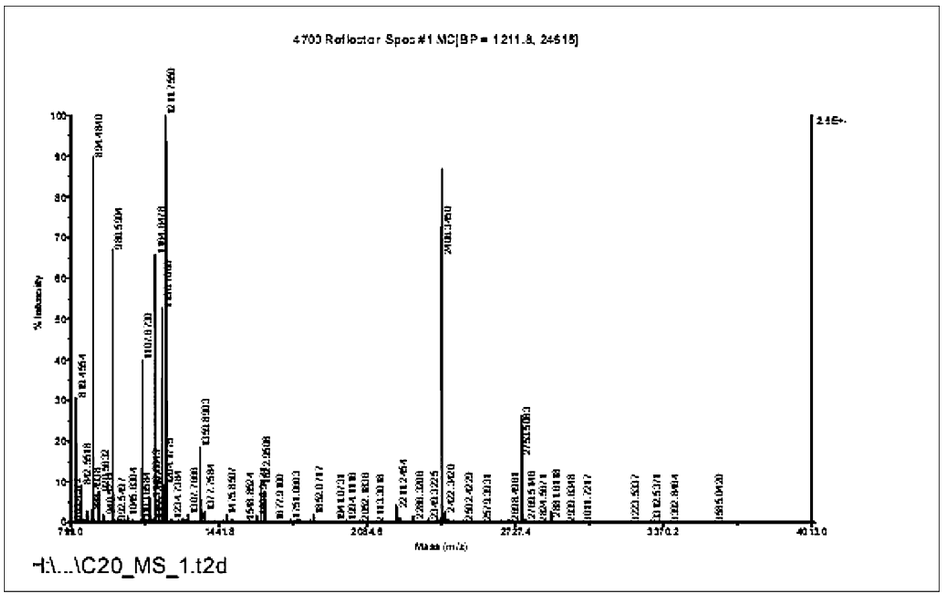
Development of cancer is a long-term and multistep process which comprises initiation, progression, and promotion stages of carcinogenesis. Many naturally occurring dietary compounds

Table: The identified proteins with differential expressions in SW480 cells treated by ITCs

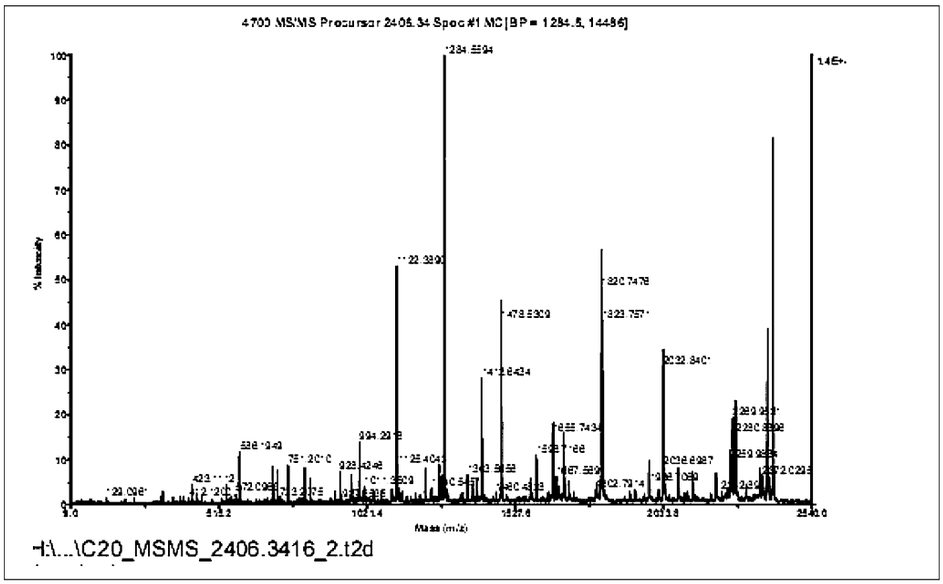
Spot no.	Protein name and function	Accession No.	Protein MW/ PI	Mascot score	Matched peptides/sequence coverage	difference
1	NME1(Nucleoside diphosphate kinase A, Pyrimidine metabolism)	IPI00375531	19640.9/5.42	793	7/49%	–2.72
2	SOD1(superoxide dismutase 1, Immunity and defense)	IPI00218733	15925.9/5.7	529	6/42%	Undetectable in ITCs-treated cells
3	STMN1(stathmin, Cell communication)	IPI00479997	17291.9/5.76	396	5/30%	–88.96
4	PPIA(peptidylprolyl isomerase A, Protein folding, Nuclear transport, Immunity and defense)	IPI00419585	18000.9/7.68	346	4/20%	3.13
5	PARK7(Parkinson disease protein, mRNA transcription regulation Stress response Cell proliferation and differentiation)	IPI00298547	19878.5/6.33	574	6/49%	−3.42
6	ACTG1(actin, gamma 1,Exocytosis, Endocytosis, Transport, Cytokinesis, Cell structure)	IPI00021440	41765.8/5.31	501	6/23%	11.31
7	GSTP1(glutathione S-transferase, Detoxification)	IPI00219757	23341/5.43	642	6/35%	4.57
8	HSPB1(heat shock 27kDa protein 1, Protein folding Stress response)	IPI00025512	22768.5/5.98	413	5/29%	2.20
9	ANXA1(annexin A1, Fatty acid metabolism, Cell surface receptor mediated signal transduction, Cell motility)	IPI00218918	38690/6.57	823	7/32%	–2.66
10	PRDX1(peroxiredoxin 1, Antioxidation and free radical removal)	IPI00000874	22096.3/8.27	672	7/47%	3.60
11	SEPT2(septin 2, Cytokinesis)	IPI00014177	41461.2/6.15	496	6/26%	−2.16
12	LDHB(lactate dehydrogenase B, Glycolysis)	IPI00219217	36615.1/5.71	567	7/28%	Undetectable in ITCs-treated cells
13	PRDX3(peroxiredoxin 3, Antioxidation and free radical removal)	IPI00374151	25822.3/7.04	314	4/18%	−2.25
14	OXCT1(3-oxoacid CoA transferase 1, Carbohydrate metabolism, Fatty acid metabolism, Coenzyme metabolism)	IPI00026516	56121.9/7.14	794	7/24%	−2.90
15	PRDX4(peroxiredoxin 4, Antioxidation and free radical removal)	IPI00011937	30520.8/5.86	612	6/32%	−1.77

from our daily consumption of fruits and vegetables have been shown to possess cancer preventive effects, such as ITCs from the crucifers. However, The upstream events by which ITCs induce apoptosis have not been fully investigated. This study is the first to investigate the antitumor activity of ITCs from broccolini seeds, especially, is the first to employ proteomic techniques to globally search for the dysregulated proteins induced by ITCs in human cancer cells.

In the present work, we firstly assessed the antitumor effect of ITCs in SW480 cells by using MTT assay, phase contrast microscope and flow cytometry, and found that ITCs induces apoptosis in SW480 cells in a dose-dependent manner. As a desirable end point for cancer therapy, apoptosis induction was probably the most potent defense against cancer, this makes ITCs be potent anti-cancer candidate agents. In fact, our data indicated that in SW480 cells ITCs *in vitro* inhibits cell proliferation stronger



(A)



(B)

1 MSSGNAK**IGH PAPNFKATAV MPDGQFK**DIS LSDYKGKYVV FFFYPLDFTF
51 VCPTEIIAFS DRAEEFKK**LN**
CQVIGASVDS HFCHLAWVNT PKKQGGLGPM
101 NIPLVSDPKR TIAQDYGVLK **ADEGISFR**GL FIIDDKGILR **QITVNDLPVG**
151 RSVDETLR**LV QAFQFTDKHG EVCPAGWKPG SDTIKPDVQK** SKEYFSKQK

(C)

Fig. 4: A representative MALDI-TOF MS peptide mass fingerprint obtained for tryptic peptides eluted from 2-D gel spot 10. The x-axis represents mass-to-charge ratio (m/z), whereas the y-axis represents relative abundance. (A) The MALDI-TOF-MS mass spectrum of PRDX1, (B) The MS/MS spectrum of peak m/z = 2406, (C) The matched peptide sequences were marked with bold font

than the chemotherapeutical agent 5-FU. Subsequently, a proteomic approach (2-DE, image analysis, and mass spectrometry with protein database interrogation strategies) was introduced to investigate protein expression patterns of SW480 cells in response to ITCs treatment. A total of 107 protein spots with 2-fold or more alterations were detected, of which 15 differentially expressed proteins were successfully identified by MALDI-TOF-MS and MS/MS followed by database search, including five up-regulated (PPIA, ACTG1, GSTP1, HSPB1, PRDX1) and 10 down-regulated (NME1, SOD1, STMN1, PARK7, ANXA1, SEPT2, LDHB, PRDX3, OXCT1, PRDX4). Most of these proteins have been implicated in human colon cancer. For example, HSPB1 is up-regulated in colon cancer cells after 5-FU treatment (Wong et al. 2008), SOD1 activ-

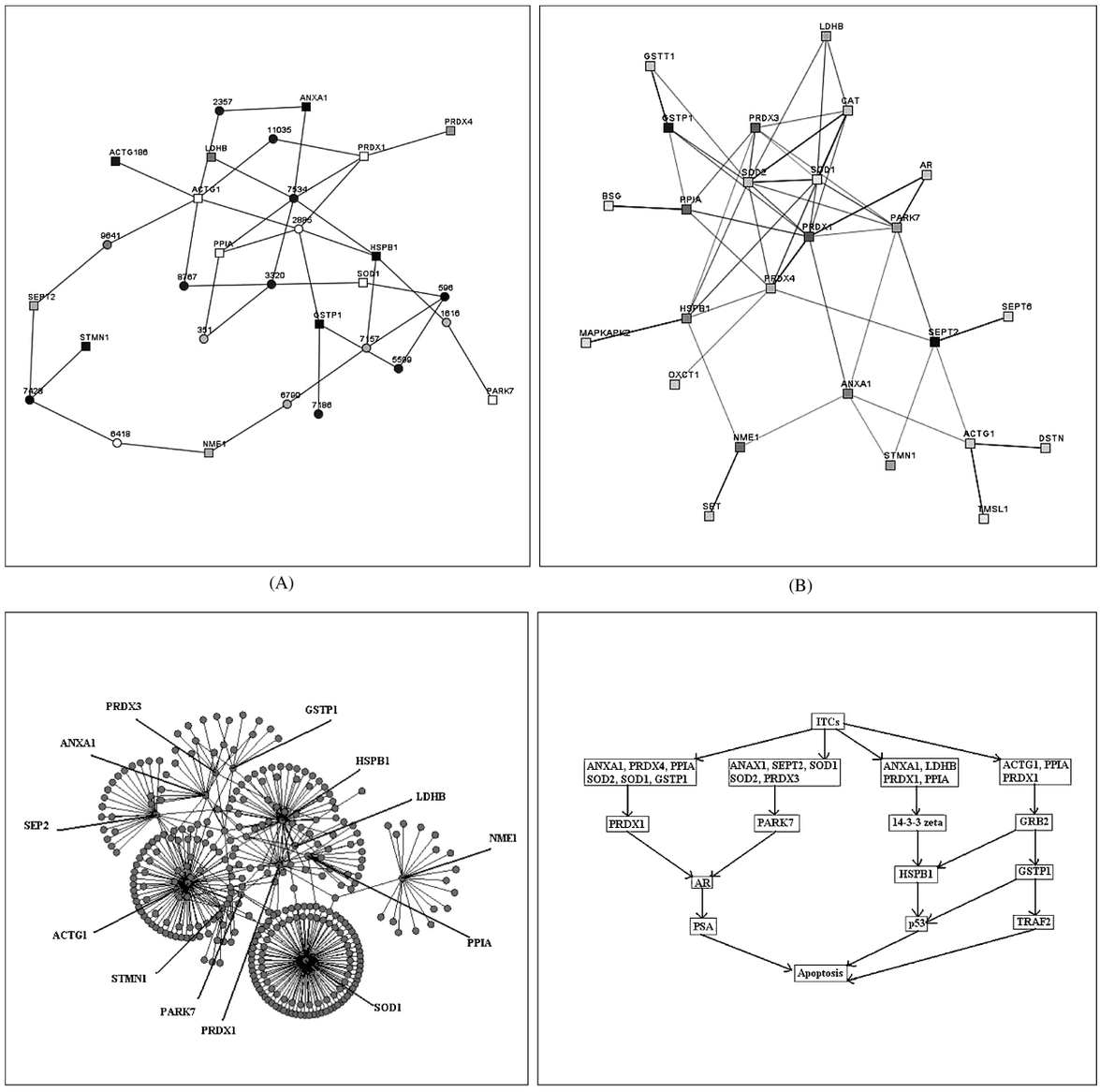


Fig. 5: (A) Protein-protein interaction networks built by KEGG spider for the identified proteins, (B) Protein-protein interaction networks built by STRING for the identified proteins, (C) Protein-protein interaction networks built by i2d for the identified proteins, (D) Predicted apoptotic pathways for ITCs treatment in SW480 cells

ity is increased after photodynamic therapy (PDT) (Kulbacka et al. 2010). However, to date, no report is available in literature about if 5 proteins (PPIA, ACTG1, PARK7, PRDX3 and OXCT1) are differentially expressed in human colon cancer (Note: ACTG1 (actin, gamma 1) is an isoform of actin, many reports in colon cancer are related to other isforms of actin), although some information about their aberrant expressions in other human cancers were documented. For example, PPIA, ACTG1 and PARK7 were found to be overexpressed in lung adenocarcinoma (Rho et al. 2009), osteosarcomas (Li et al. 2010), and cholangiocarcinoma (Kawase et al. 2009), respectively. PRDX3 has a significant role in cell cycle regulation and could be a potential proliferation marker in breast cancer (Chua et al. 2010). Our data showed that ACTG1 and PPIA are increased by 11.3- and 3.1-fold respectively, while PARK7, PRDX3 and OXCT1 are decreased by 3.4-, 2.2- and 2.9-fold respectively, in ITCs-treated colon cancer cells. It merits further investigation of their roles in human colorectal carcinogenesis and in the development of diagnostic or therapeutical biomarkers.

Furthermore, bioinformatic analysis was employed to infer the protein networks associated with the identified 15 proteins. By network analysis, some very interesting interactors were identified, including GRB2, TRAF2, TP53, AR, BCL2, and 14-3-3 zeta. In particular, among the 15 identified proteins, 5,5 and 8 proteins are the direct interactors of GRB2, 14-3-3 zeta or SOD2, respectively. This suggests that they could play important roles in mediating ITCs-induced apoptosis in colon cancer cells.

Finally, in order to find some clues about how these interactors specifically mediate ITCs to induce apoptosis of SW480 cells, we searched these interactors in the cancer pathway map provided by KEGG database (map09020) and found that there are several routes to apoptosis endpoint: (1) TRAF2-apoptosis; (2) p53-apoptosis; (3) AR-PSA-apoptosis. Thus, combining them with the results of network analysis, three potential pathways for

ITCs-induced apoptosis in colon cancer cells could be predicted (Fig. 5D). We speculated that the inhibitions of these pathways might represent several new strategies for treating colon cancer, the future work will test these predictions.

4. Experimental

4.1. Materials

ITCs were prepared by hydrolysis of Broccolini seeds extracts in our laboratory, primarily consisting of 4 ITCs components (contents up to 80%): SFN (19%), PEITC (23%), BITC (11%), and 1-isothiocyanato-butane (27%). Human colon cancer cell line SW480 was purchased from the people hospital of Guangdong province (Guangzhou, China). Complete DMEM media was obtained from GIBCO (Grand Island, NY, USA). Glycerol, thiourea, and trypsin (sequencing grade) were purchased from Sigma (St. Louis, MO, USA). TEMED was obtained from Promega (Madison, WI, USA). Dithiothreitol (DTT), acrylamide, methylenebisacrylamide, CHAPS, Clean-up kit, 2D Quant kit were obtained from Amersham Biosciences-GE Healthcare (Uppsala, Sweden). Immobiline pH-gradient (IPG) strip (pH 4-7), Pharmalyte (pH 4-7), IPG buffer (pH 4-7) were obtained from Bio-Rad. All other chemicals and solvents were of the highest purity available.

4.2. Cell culture and MTT assay

For routine maintenance, SW480 cells were cultured in suspension in DMEM media with 10% fetal bovine serum (GIBCO) in a water-jacketed 5% CO_2 incubator at 37 °C (Forma Scientific, Marietta, OH).

Cell growth inhibition was evaluated using a standard colorimetric MTT assay. Briefly, cells in logarithmic growth phase were plated in 96-well microtitre plates at a density of $3 \times 10^3 \sim 10^4$cells/100 μl/well and allowed to incubate 24 h at 37 °C for attachment. Subsequently, Broccolini seeds ITCs samples and chemotherapeutical agent 5-FU (5-flurouracil, a standard chemotherapeutic drug to treat colorectal cancer) were diluted with complete medium to the desired initial concentration (10, 30, 60, 90, 120 μg/mL) and then added to cells of the logarithmic phase. After incubation for another 24 h, the MTT solution (Bornem, Belgium) (20 μl of 5 mg/ml) was added to each well, then for additional 4 h incubation at 37 °C. MTT-containing medium were removed and the formazan crystals, formed within the cells, were solubilized by the addition of DMSO (Bornem, Belgium) (150 μl/well) and agitation. The absorbance of the samples and control cultures were read at 490 nm with a spectrophotometer (Model 550 Microplate Reader). The half inhibition rate IC50 was calculated. Each experiment for cell lines exposed to various concentrations of ITCs was in triplicate.

4.3. Morphology and flow cytometry analysis of ITCs treated SW480 cells

Cells in the logarithmic growth phase were plated in 24-well microtitre plates at a density of 1.5×10^4 cells/500 μl/well and allowed to incubate 24 h at 37 °C for attachment. After treated with ITCs (10, 30, 60, 90, 120 μg/mL) and incubated for 24 h, the cellular morphology was observed using phase contrast microscopy (Leica, Wetzlar, Germany).

Flow cytometry was introduced to further investigate cells apoptosis effect by ITCs. The cells were seeded in 100 mm culture dishes at a density of 2×10^6 and allowed to attach overnight. The medium was replaced with fresh complete medium containing the desired concentration of ITCs (10, 30, 60, 90, 120 μg/mL), and the cells were incubated at 37 °C for 24 h followed by harvested by centrifugation (200 × g, 5 min). The collected cells were washed with cold phosphate-buffered saline (PBS) for three times and fixed in cold 75% ethanol at 4 °C for 24 h. After that, the cells were rinsed with PBS two times and mixed with an equal volume of double staining solution (Annexin V-FITC and PI, 50 μg/ml RNase and 0.1% (w/v) Triton X-100 in sodium citrate (3.8 mM)). Apoptosis were measured using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The percentages of apoptotic (Annexin+/PI-) and necrotic (Annexin+/PI+) cells were determined with Cell Quest Pro software.

4.4. Proteomic sample preparation

Cultured cells were washed three times in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) after removal of the medium. Then cells were directly disrupted in 300 μl of a lysis buffer (7 M urea, 4% CHAPS, 2 M thiourea, 30 mM Tris, pH 8.8) containing 1% protease inhibitors, 50 μg/ml RNase, and 200 μg/ml DNase. After pelleting the insoluble material by centrifugation at 15,000 × g for 30 min at 4 °C, the supernatant was collected. According to the manufacture's instructions, the proteins samples were cleaned up and quantified with Clean-up kit and 2D Quant Kit.

4.5. Two-dimensional gel electrophoresis

For the first dimensional isoelectric focusing (IEF), the protein solutions (450 μg) were directly applied onto IPG strips (24 cm, pH 4-7, linear), and rehydrated at 17 °C and 50 V for 12 h. After rehydration, isoelectric focusing was carried out using a Bio-Rad PROTEAN IEF cell (Bio-Rad) and focusing was conducted by stepwise increase of the voltage as follows: the initial voltage was held at 250 V for 30 min; in the second step was quickly increase from 250 to 1000 V within 60 min; in the third step was linearly increased from 1000 to 10,000 V within 5 h; for the last step it was maintained at 500 V for 10 h. The plate temperature was maintained at 20 °C during isoelectric focusing. After IEF separation, each IPG strip was incubated in equilibration buffer I (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 1% DTT) for 20 min with gentle agitation, followed by incubation in buffer ∏ (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2.5 % IAA) for 15 min with gentle agitation. IPG strips were then rinsed with SDS-PAGE running buffer and were directly loaded onto 11–17% linear acrylamide gradient gels. Subsequently, strips were overlayed with agarose solution (60 mM Tris-HCl pH 6.8, 60 mM SDS, 0.5% (w/v) agarose, 0.01% (w/v) bromophenol blue). The second dimensional separation was performed in two steps at 10 °C: 15 mA/gel for 30 min and 30 mA/gel until the bromophenol blue dye front reached the bottom of the gel. Then SDS-PAGE gels were washed with deionized water three times 15 min each and stained for at least 12 h with Coomassie Brilliant Blue G-250 (20% colloidal Coomassie ethanol, 1.6% phosphoric acid, 8% ammonium sulfate, 0.08% Coomassie Brilliant Blue G-250). Three replicates were run for the samples.

4.6. 2-DE Image analysis

The 2-DE images were scanned using a Typhoon laser scanner (GE Healthcare, Uppsala, Sweden) at the resolution of 100 μm and analyzed using PDQuest package software version 7.1.1 (Bio-Rad, Hercules, CA, USA). Spot detection, background subtraction and spot quantitation were performed on 16-bit TIEF images acquired with a scanning densitometer. The protein spot volume was measured according to the lowest boundary mode of background selection. The spot volumes were normalized according to the total spot volume for each gel. Protein spot intensity was defined as the normalized spot volume which is the ratio of the single spot volume to the total spots volumes on a 2-DE. The computer analysis allowed automatic detection and quantification of protein spots, as well as matching between control gels and gels from treated samples. The significance of differences of protein spots were evaluated by Student's t-test and $p < 0.05$ was considered as significant.

4.7. In-gel tryptic digestion

The protein changed spots were manually excised and transferred to 500 μL siliconized Eppendorf tubes. Subsequently, the gel plugs destained twice with freshly prepared 200 μL of 50% (v/v) acetonitrile/25 mM ammonium bicarbonate buffer and incubated at room temperature (25 °C). The gel plugs were then washed once with 200 μL of 100% ACN for 5 min at room temperature and dried in a Speed Vac Plus SC110A (Savant, Holbrook, NY, USA) vacuum concentrator. After the plugs had dried completely, 20 μL of 25 mM ammonium bicarbonate containing 5 ng of sequencing grade modified trypsin solution (Promega, Madison, WI) was added and the samples incubated at 37 °C for at least 16 h for complete digestion. Peptides were extracted from the gel plugs with 50 μL of 50% (v/v) ACN/1% (v/v) TFA and agitation in a shaker (140 rpm) for 15 min. Tryptic peptides were collected, dried, and resuspended in 4 μL of 20% ACN/0.1% TFA for MS analysis.

4.8. Protein identification

Equal volumes of trypsinized samples (0.5 μl) and the matrix solution (0.5 μl) containing 5 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Fluka, St. Louis, MO) prepared in 30% ACN, 0.1% v/v TFA and 2% w/v ammonium citrate. The samples/matrix mixture was then spotted onto MALDI-TOF MS target plate (384-well AnchorChip). Peptide extracts were analyzed on a ABI 4800 MALDI-TOF/TOF-MS (Applied Biosystems, Foster City, CA) in the positive ion reflectron mode. The standard peptide mixture (904.458 gradykinin 1296.685 angiotensin 1570.677 Glul-fibrinopeptide 2093.08, ACTH (1–17); 2465.199, ACTH (18-39); 3657.929, ACTH (7–38)) was used as external calibration. We used a protein molecular mass range of 800∼4000 Da and a mass tolerance of 100 ppm for the internal calibration. Top 5 precursor ions with S/N > 50 were subject to further MS/MS analysis. Both the MS and MS/MS data were interpreted and processed by using the GPS Explorer software (V3.6, Applied Biosystems), then the obtained MS and MS/MS spectra per spot were combined and submitted to MASCOT search engine (V2.1, Matrix Science, London, U.K.) by GPS Explorer software. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 65 were considered statistically significant ($p \leq 0.05$). The individual MS/MS spectrum with statistically significant

($p \leq 0.05$) best ion score (based on MS/MS spectra) was also accepted. The search parameters were as follows: (1) IPI-human database; (2) maximum missed cleavage 1; (3) no fixed modifications; (4) variable modifications, cysteine carboamido methylated and methionine oxidized; (5) peptide mass tolerance, 30–60 ppm; (6) fragment mass tolerance, 0.2–0.3Da.

4.9. Bioinformatics Analysis

The molecular function and biological process of differentially expressed proteins were defined by PANTHER (Proteins Through Evolutionary Relationships) (Thomas et al. 2003). Protein-protein interaction networks were built by PPI Spider (Antonov et al. 2009), STRING (http://string.embl.de/) and i2d (Brown and Jurisica, 2007).

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