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Pharmacodynamics of dietary phytochemical indoles I3C and DIM: Induction of Nrf2-mediated phase II drug metabolizing and antioxidant genes and synergism with isothiocyanates

Constance Lay-Lay Saw[a,b,†], Melvilí Cintrón[a,c,†], Tien-Yuan Wu[a,d], Yue Guo[a,e], Ying Huang[a,d], Woo-Sik Jeong[f] and Ah-Ng Tony Kong[a,b,d,*]

[a]*Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA*
[b]*Center for Cancer Prevention Research, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA*
[c]*Department of Industrial Biotechnology, University of Puerto Rico in Mayagüez, Calle Post, Mayagüez, PR 00681-9000*
[d]*Graduate Program in Pharmaceutical Sciences, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA*
[e]*School of Pharmacy, China Pharmaceutical University, Nanjing, 211198, People's Republic of China*
[f]*School of Food and Life Sciences, Inje University, 607 Obang-dong, Gimhae, 621-749, South Korea*

ABSTRACT: The antioxidant response element (ARE) is a critical regulatory element for the expression of many phase II drug metabolizing enzymes (DME), phase III transporters and antioxidant enzymes, mediated by the transcription factor Nrf2. The aim of this study was to examine the potential activation and synergism of Nrf2-ARE-mediated transcriptional activity between four common phytochemicals present in cruciferous vegetables; the indoles: indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM); and the isothiocyanates (ITCs): phenethyl isothiocyanate (PEITC) and sulforaphane (SFN). The cytotoxicity of the compounds was determined in a human liver hepatoma cell line (HepG2-C8). The combination index was calculated to assess the synergistic effects on the induction of ARE-mediated gene expressions. Quantitative real-time polymerase chain reaction (qPCR) was employed to measure the mRNA expressions of Nrf2 and Nrf2-mediated genes. I3C and DIM showed less cytotoxicity than SFN and PEITC. Compared with I3C, DIM was found to be a stronger inducer of ARE. Synergism was observed after combined treatments of 6.25 μM I3C + 1 μM SFN, 6.25 μM I3C + 1 μM PEITC and 6.25 μM DIM + 1 μM PEITC, while an additive effect was observed for 6.25 μM DIM + 1 μM SFN. Induction of endogenous Nrf2, phase II genes (*GSTm2*, *UGT1A1* and *NQO1*) and antioxidant genes (*HO-1* and *SOD1*) was also observed. In summary, the indole I3C or DIM alone could induce or syngergistically induce in combination with the ITCs SFN or PEITC, Nrf2-ARE-mediated gene expression, which could potentially enhance cancer chemopreventive activity.

Key words: antioxidant response element (ARE); nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2); indole-3-carbinol (I3C); 3,3′-diindolylmethane (DIM); isothiocyanates

Introduction

When cells are exposed to excessive oxidative stress, DNA can go through oxidative damage [1]. When coupled with chronic inflammation [2] with

*Correspondence to: Rutgers, The State University of New Jersey, Ernest Mario School of Pharmacy, Room 228, 160 Frelinghuysen Road, Piscataway, NJ-08854, USA.
E-mail: kongt@pharmacy.rutgers.edu
†These authors contributed equally to this work.

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the formation of DNA adducts, this would lead to enhanced genomic instability, neoplastic transformation, and ultimately drive cancer formation and tumorigenesis [3]. To counteract oxidative stress, induction of various cellular protective enzymes including phase II drug metabolizing enzymes (DME), phase III transporters and antioxidant enzymes occur [4,5]. Carcinogens are typically metabolized via oxidation and reduction by phase I DME [6]. The resulting products will subsequently undergo phase II conjugations catalysed by phase II DME such as glutathione *S*-transferases (GST) and UDP-glucuronosyltransferases (UGT), resulting in the formation of conjugated products that are more water soluble and can be excreted easily in the bile or in the urine [6,7].

The induction of phase II DME can be attributed largely to the transcriptional control of the antioxidant response element (ARE) by the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2) [8]. Nrf2 is known as a key regulator of the ARE-mediated gene expression and therefore a potential target for cancer chemopreventive compounds [9–11]. Nrf2 is inhibited in the cytoplasm by the anchor protein Kelch-like ECH-associated protein-1 (Keap1) and in the presence of oxidative stress or chemical inducers, Nrf2 is released from Keap1 inhibition, translocates to the nucleus, dimerizes with small Mafs (sMaf) and binds to ARE consensus sequence [12]. Regulation of Nrf2 by cancer chemopreventive compounds would lead to the induction of phase II DME, phase III transporters and antioxidative stress enzymes such as heme oxygenase 1 (HO-1). HO-1 catalyses the degradation of heme to carbon monoxide, iron and biliverdin. HO-1 is critically essential in cellular defensive mechanisms and is implicated with various pathophysiological disease conditions including inflammation, atherosclerosis, neurodegenerative diseases and cancers [13].

The cruciferous vegetables contain abundant phytochemicals with potentially super cancer chemopreventive activities [14]. Cruciferous vegetables, including broccoli, Brussels sprouts, cabbage and cauliflower, are rich in glucosinolates that can endogenously be converted into compounds including indoles [indole-3-carbinol (I3C) and 3,3′-diindolylmethane (DIM)] and isothiocyanates (ITCs) [phenethyl isothiocyanate (PEITC) and sulforaphane (SFN)] (Figure 1) upon ingestion [14]. Epidemiological studies indicate that dietary consumption of these compounds by a high consumption of cruciferous vegetables would reduce cancer risk [15–17].

Phytochemicals, including indoles and ITCs possess potent cancer chemopreventive effects [18–21]. Previous studies showed that the indoles could achieve the cancer chemopreventive effects potentially via multi-targets. For instance, they are capable of inducing antioxidant activity, regulate cellular proliferative genes, induce cell cycle arrest/apoptosis, regulate hormone metabolism and stimulate the immune system [22–28]. ITCs can also elicit their chemopreventive effects via various mechanisms such as regulating DME

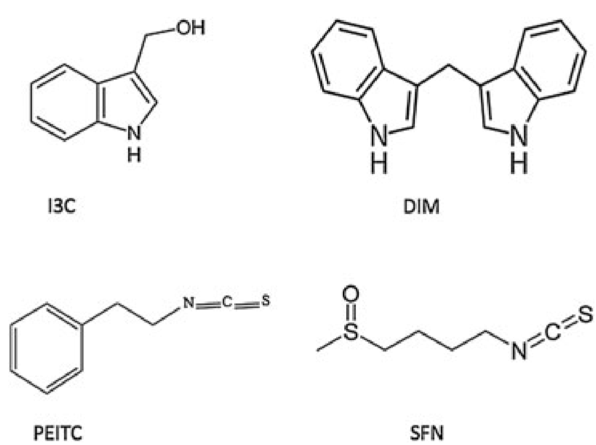

Figure 1. Chemical structures of phytochemicals used in the current study

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phase I cytochrome P450s and phase II DME, Nrf2-Keap1 antioxidative stress and antiinflammatory NFkB pathways, as well as inducing cell cycle arrest/apoptosis [20,21].

The Nrf2-ARE signaling pathway has been shown to play an important role in cancer chemoprevention [9–11]. The present study investigated the transcriptional activation of the Nrf2-ARE signaling pathway by the indoles (I3C and DIM) and the potential synergistic effect between the indoles and the ITCs (SFN and PEITC). To accomplish these goals, the human liver carcinoma cell line was utilized (HepG2-C8-ARE-luciferase cells; the original HepG2 cell stabilized with the ARE-luciferase reporter gene [29]), a metabolic competent cell line, which is a useful *in vitro* cell culture model to study the regulation of DME [29]. Our results show that the indoles, I3C and DIM, alone can transcriptionally activate Nrf2-ARE-mediated gene expression, and importantly, the indoles can also act synergistically in activating Nrf2-ARE-mediated signaling when combined with the ITCs, SFN or PEITC.

Materials and Methods

Materials

The I3C, DIM and PEITC were purchased from Sigma Chemicals Co. (St Louis, USA). The SFN was obtained from LKT Laboratories (St Paul, USA).

Cell culture

The stably transfected single clone HepG2-ARE-C8 (HepG2-C8) cell line has been established previously in our laboratory using the *pARE-TI-luciferase* reporter gene [29–36]. The cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 1.17 g/l sodium bicarbonate, and 100 unit/ml penicillin, 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2.

MTS assay

The cytotoxicity of the phytochemicals was tested in HepG2-C8 cells using the CellTiter 96 aqueous non-radioactive cell proliferation MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison, WI). The cells were first cultured in 96-well plates for 24 h and then were treated with I3C, DIM, PEITC or SFN at various concentrations for 24 h. The cells were then treated with MTS for 1 h at 37 °C. Absorbance of the formazan product was read at 490 nm with a μQuant Biomolecular Spectrophotometer from Bio-Tek Instruments Inc. (Winooski, VT). Independent control studies were conducted using 1% and 10% FBS medium.

ARE-luciferase assay

The HepG2-C8 cells were cultured in 12-well plates and each well contained 1 million cells in 1 ml of 10% FBS medium. The cells were treated with compounds for 24 h. The luciferase activity was determined using a luciferase kit from Promega (Madison, USA) according to the manufacturer's instructions. Briefly, after treatments for 24 h, the cells were washed twice with ice-cold phosphate buffered-saline (PBS, pH 7.4) and harvested in 1× reporter lysis buffer and kept at −20 °C overnight. After centrifugation at 4 °C, 12000 rpm for 5 min, a 10 μl aliquot of the supernatant was assayed for luciferase activity with a Sirius luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The luciferase activity was normalized against protein concentration, determined by a BCA protein assay (Pierce, Rockford, USA), and expressed as the fold induction over the luciferase activity of control vehicle-treated cells. At least two to three independent studies were conducted in triplicates.

RNA extraction and quantitative real-time PCR

The cells were treated similarly to the MTS and ARE-luciferase assays described above using 10% FBS medium. The incubation of the compounds with the cells was terminated 6 h later. The mRNA expression was evaluated utilizing a quantitative real-time polymerase chain reaction (qPCR). An RNeasy kit from Qiagen was used for RNA extraction (Valencia, CA). The total RNA was reverse-transcribed to cDNA by TaqMan Reverse Transcription Reagents (Applied Biosystems Inc, Foster City, CA). SYBR Green (Applied Biosystems Inc, Foster City, CA)

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fluorescence was used to measure the product of qPCR. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the housekeeping gene, and the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems Inc, Foster City, CA) was used as described previously [37] to detect quantitatively the induction of mRNA of Nrf2, phase II DME GSTm2, NAD(P)H dehydrogenase, quinone 1 (NQO1), UGT family, polypeptide A1 (UGT1A1) and antioxidant enzymes HO-1, superoxide dismutase 1 (SOD1). The primer pairs were designed using the Primer Quest Oligo Design and Analysis Tool by Integrated DNA Technologies Inc. (Coralville, IA, USA) and the sequences are listed in Table 1. At least four wells of each treatment were performed and duplicate samples were carried out for each treatment.

Western blotting

The cells were treated similarly to the MTS, ARE-luciferase and qPCR assays described above using 10% FBS medium. The HepG2-C8 cells were treated with the compound for 24 h. The cells were washed with ice-cold PBS (pH 7.4) and harvested in cell culture lysis reagent (Promega E153A, Madison, WI). The homogenate was centrifuged at 4 °C, 12000 rpm for 5 min. The supernatants were collected and 15 μg of total protein, as determined by BCA protein assay (Pierce, Rockford, USA), was mixed with 5 μl Laemmli's SDS-sample buffer (Boston Bioproducts, Ashland, MA, USA) and denatured at 95 °C, for 5 min. The samples and the protein standard (Bio-Rad, Hercules, CA, USA) were then loaded onto a polyacrylamide gel (Criterion Tris–HCl gel, Bio-Rad Lab, Hercules, CA, USA) and gel electrophoresis was run at 130 mA for 60 min. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA) over 1.5 h using a semi-dry transfer system (BioRad, Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) solution for 1 h at room temperature and incubated with the primary antibody (1:1000, in 3% BSA in Tris-buffered-saline and Tween 20, TBST) overnight at 4 °C. Antibody against actin (catalog no. sc-1616) and SOD1 (catalog no. sc-11407) were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., CA, USA). Antibody against Nrf2 (catalog no. 2178–1) was purchased from Epitomics (Burlingame, CA, USA). After hybridization with primary antibody, the membranes were washed with TBST four times. The immunoreactions were continued with the respective secondary antibodies (1:5000, in 3% BSA in TBST) purchased from Santa Cruz Biotechnology, Inc., CA, USA, for 1 h at room temperature. After washing four times with TBST, the immunocomplexes were determined using the enhanced chemiluminescent system to detect horseradish peroxidase on the immunoblots (Thermo Scientific, Rockford, IL, USA) and the bands were visualized and captured by a BioRad ChemiDoc XRS system (Hercules, CA, USA).

Table 1. Human oligonucleotide primers used for qPCR

Gene	Association no.	Forward (5′) primer	Reverse (3′) primer
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	NM_002046.3	5′-TCG ACA GTC AGC CGC ATC TTC TTT-3′	5′-ACC AAA TCC GTT GAC TCC GAC CTT-3′
Glutathione *S*-transferase mu 2 (*GSTm2*)	NM_000848	5′-ACT AAA GCC AGC CTG ACC TTC CTT-3′	5′-AAT GCT GCT CCT TCA TGC AAC ACG-3′
Heme oxygenase-1 (*HO-1*)	NM_206866	5′-ACG CGT TGT AAT TAA GCC TCG CAC-3′	5′-TTC CGC TGG TCA TTA AGG CTG AGT-3′
NAD(P)H dehydrogenase, quinone 1 (*NQO1*)	NM_001025434	5′-AAG GAT GGA AGA AAC GCC TGG AGA-3′	5′-GGC CCA CAG AAA GGC CAA ATT TCT-3′
Nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*)	NM_001145413	5′-TGC TTT ATA GCG TGC AAA CCT CGC-3′	5′-ATC CAT GTC CCT TGA CAG CAC AGA-3′
Superoxide dismutase 1 (*SOD1*)	NM_000454	5′-GCA GGG CAT CAT CAA TTT CGA GCA-3′	5′-TGC AGG CCT TCA GTC AGT CCT TTA-3′
UDP-Glucuronosyltransferase 1 family, polypeptide A1 (*UGT1A1*)	NM_000463	5′-ATG ACC CGT GCC TTT ATC ACC CAT-3′	5′-AGT CTC CAT GCG CTT TGC ATT GTC-3′

Combination index calculation

To determine the synergistic effect between the combination of two different compounds, the combination index (*CI*) was calculated with the following formula: $CI = d1/Dx_{,1} + d2/Dx_{,2}$ where $d1$ and $d2$ are doses of drugs 1 and 2 in combination, which produces an effect x. $Dx_{,1}$ and $Dx_{,2}$ are the doses of drug 1 and 2 that produce the same effect x when given alone. When the *CI* is equal to, less than or greater than 1, the combination dose will be additive, synergistic or antagonistic, respectively, as described previously [37,38]. This approach is based on the Loewe additivity model and although the exact mechanism of interaction may be unknown, this model is one of the most commonly used reference models for evaluating potential drug–drug interactions [39]. Using this *CI* calculation for the ARE-luciferase activity induced by I3C or DIM combined with PEITC or SFN, it is possible to identify whether the combination of these phytochemicals at certain concentrations would be synergistic, antagonistic or additive.

Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). MTS assay data were analysed using one-way ANOVA with a post hoc multiple comparison analysis by Bonferroni. Luciferase assay and qPCR data were analysed statistically using Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

Results

Cell viability by MTS assay

To test the cell viability of I3C, DIM, SFN and PEITC, the MTS assay was employed. DIM and I3C showed less toxicity than SFN and PEITC in 1% FBS medium (Figure 2), SFN and PEITC showed similar cell viability inhibitory concentrations (IC_{50}) of around 20 µM, whereas I3C and DIM had a higher IC_{50} of 135 µM and 51 µM, respectively. Using 10% FBS, several previous publications showed that DIM was more cytotoxic than I3C, hence the same dosage was tested in HepG2-C8 cells with 10% FBS. The cytotoxicity of HepG2-C8 was affected more with DIM than I3C, i.e. DIM showed an IC_{50} of around 85 µM, while I3C showed an IC_{50} of 300 µM in 10% FBS medium (data not shown).

ARE-luciferase activity

In the ARE transcriptional activation assay, the cells were treated with higher doses of DIM and I3C (25 and 75 µM), since from the MTS assay the viability was not affected at these concentrations in 10% FBS medium (data not shown). To evaluate the transcriptional activation of ARE, an ARE-luciferase reporter assay was performed [40]. SFN and PEITC were used as positive controls and 0.1% DMSO was used as a negative control. The ARE-luciferase activity was expressed as the fold induction over the negative vehicle control. All compounds alone and in combinations induced ARE-luciferase activity in HepG2-C8 cells with different potency (Figure 3). DIM at 75 µM strongly induced the ARE-luciferase compared with any other treatment ($p < 0.05$). Interestingly, 25 µM DIM with 1 µM SFN (DIM25/SFN1) was synergistic but not for 25 µM I3C with 1 µM SFN (Figure 3). Although there were three synergistic interactions at low doses of combination having ARE activities close to value 1, all the *CI* were <1, and $p < 0.05$. Specifically, synergistic effects were observed for the combinations of 6.25 µM I3C with 1 µM SFN (I3C6.25/SFN1, *p* value for $CI = 0.045$), 6.25 µM I3C with 1 µM PEITC (I3C6.25/PEITC1, *p* value for $CI = 0.044$) and 6.25 µM DIM with 1 µM PEITC (DIM6.25/PEITC1, *p* value for $CI = 0.003$). An additive effect was observed with 6.25 µM DIM with 1 µM SFN (DIM6.25/SFN1), whereas 25 µM I3C with 1 µM SFN (I3C25/SFN1) was antagonistic. The DIM25/SFN1 treatment displayed the most synergism, and I3C6.25/SFN1, DIM6.25/PEITC1 and I3C6.25/PEITC1 were not so obvious, however, their *CI* values were less than 1 (i.e. synergistic). DIM6.25/SFN1 had a *CI* value of 1 (i.e. additive). I3C25/SFN1 had a *CI* value of more than 1 (i.e. antagonistic). The classification of synergistic, additive or antagonistic was based mathematically on the *CI* calculations that were derived from the dose response of a single compound, and the response of the combinations at different doses. The effects of using a different cell density at similar drug concentrations in medium with

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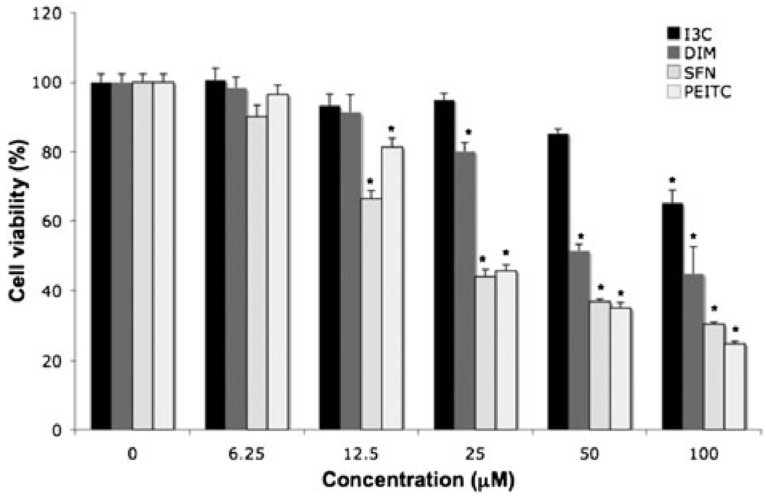

Figure 2. Effect of the compounds tested on the cell viability determined by MTS assay, using medium with 1% FBS. Results are expressed as the mean ± SEM. $^*p<0.05$, compared with corresponding value for 0.1% DMSO-treated cells

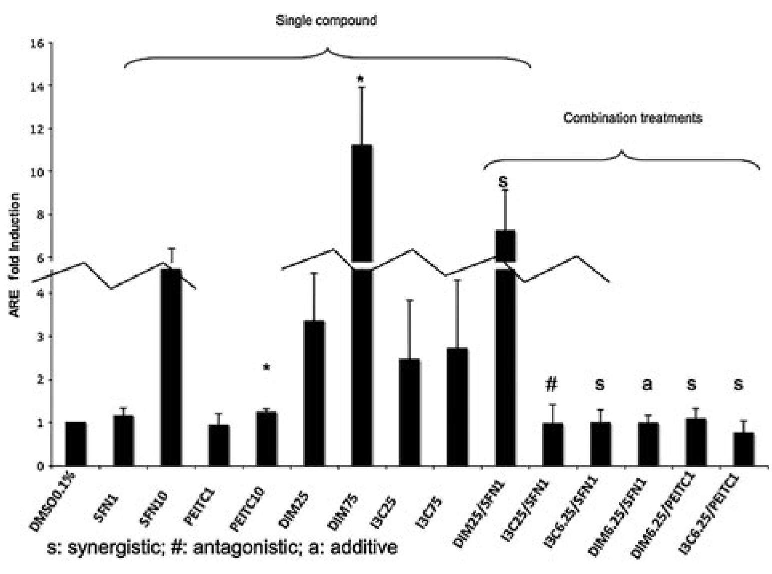

Figure 3. Luciferase activity in HepG2-C8 cells. All combinations are described in Materials and Methods. 's' denotes synergistic; '#' denotes antagonistic; 'a' denotes additive, though DIM6.25/PEITC1 is considered additive as the combination index (*CI*) is around 1, most of the mRNA levels were synergistically induced by this combination (see Figure 4). When doses of I3C and DIM lower than 25 μM were tested, no significant induction was observed. Therefore the single dose of 6.25 μM I3C and DIM is not presented. The *CI* for the combination studies was calculated as published previously to determine synergistic, additive or antagonistic effects [37,39]. The changes in the fold induction for synergistic and additive combination may not appear as robust, nonetheless the qPCR results verify the findings (see Figure 4). The broken lines break the relatively higher fold changes into two corresponding connecting bars; the relative folds across all groups are maintained

1% FBS gave similar observations (data not shown). When doses of I3C and DIM lower than 25 μM were tested in 10% FBS medium, no significant induction was observed (data not shown). As there was an obvious dose response for single treatment with DIM (i.e. DIM25 and DIM75), and not for I3C25 and I3C75, however, the *CI* calculations for DIM25/SFN1 and I3C25/SFN1

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showed *CI* of 0.7 and 3, respectively. Next, the study verified the identified additive/synergistic combinations, particularly at those lower concentrations that may be more physiologically relevant concentrations of indoles and ITCs using quantitative real-time polymerase chain reaction (qPCR) and western blotting analyses for the Nrf2-ARE-mediated genes, as described below.

qPCR

To confirm that the cells treated with the agents induced endogenous phase II DME and antioxidant genes, qPCR was conducted to quantify the mRNA expression. Values higher than 1 were considered positive in comparison with cells treated with control 0.1% DMSO. The results for the induction of Nrf2, phase II DME and antioxidant genes are shown in Figure 4. I3C alone at 25 μM did not show significant induction of *Nrf2* and *HO-1* mRNA (Figure 4A and 4B). On the other hand, 25 μM DIM showed about a 3 fold-induction for both of these genes. The higher dose, 75 μM DIM, induced only *Nrf2* and *HO-1* gene expression (Figure 4A and 4B), which was somehow not correlated to the dose-dependency activation of the ARE-luciferase above (Figure 3). Interestingly, *NQO1* gene expression was not significantly induced by SFN at any concentration, but it was greatly induced by PEITC even at a very low concentration of 1 μM (Figure 4C). Similar to 10 μM PEITC, increasing the concentrations of I3C and DIM from 25 μM to 75 μM, did not enhance *NQO1* gene expression any further (Figure 4C). The time course study using SFN and PEITC at 6 h treatment indicated that the lower concentration of PEITC was a faster ARE inducer compared with SFN at 6 h. In addition, 1 μM PEITC induced higher ARE activity than 10 μM PEITC (data not shown). At 12 h, both SFN and PEITC at 10 μM had higher ARE induction than at the lower 1 μM concentration (data not shown). It is postulated that these observations possibly could be due to additional different mechanisms by which SFN and PEITC regulate gene expression, in addition to the common Nrf2-ARE mediated signaling pathway [21] and this is further discussed below.

Among the combination treatments, 6.25 μM DIM with 1 μM PEITC had the greatest induction of *SOD1* (Figure 4D) and *UGT1A1* (Figure 4E), whereas 6.25 μM DIM with 1 μM SFN induced *GSTm2* the most (Figure 4F). These results confirmed the synergistic and additive effects of the combinations generated from the ARE-luciferase studies, respectively. In comparison with the other genes, with the same combinations, synergism was observed for *HO-1*, which was induced the most (Figure 4B).

Western blot

Figure 5 shows the selected protein biomarkers of Nrf2 and one of the Nrf2-downstream targets, SOD1, was examined using western blotting. It was hypothesized that the combination of low doses of indoles and ITCs could enhance Nrf2/ARE-mediated Nrf2 and Nrf2-target antioxidant enzymes such as SOD1. I3C and DIM alone at various concentrations was able to induce the protein levels of Nrf2 and SOD1 in a dose-dependent manner (Figure 5). The combinations of *low doses* of indoles and ITCs were also able to induce higher protein expression of SOD1 compared with the individual agent at higher concentrations and higher induction of Nrf2 and SOD1 proteins was also observed (Figure 5, representative of three separate experiments with similar results), which corroborated the synergistic effects ($CI < 1$) for the combination treatments identified in the ARE-luciferase assay (Figure 3). In contrast, Nrf2 protein expression for DIM6.25/SFN1 treatment which was shown as additive using the *CI* calculation (Figure 3), showed slightly less than 1 but yet the SOD1 expression was almost 2 fold compared with the 0.1% DMSO control. These results suggest that differential signaling pathways were activated by the indoles and the ITC at different concentrations with different combinations and that sometimes, endogenous gene expression would vary from the simple single promoter transcriptional reporter gene assay, so that the latter would provide a quick screen for potential *in vivo* activities.

Discussion and Conclusion

In general, disease prevention including cancer chemoprevention could conceivably be achieved

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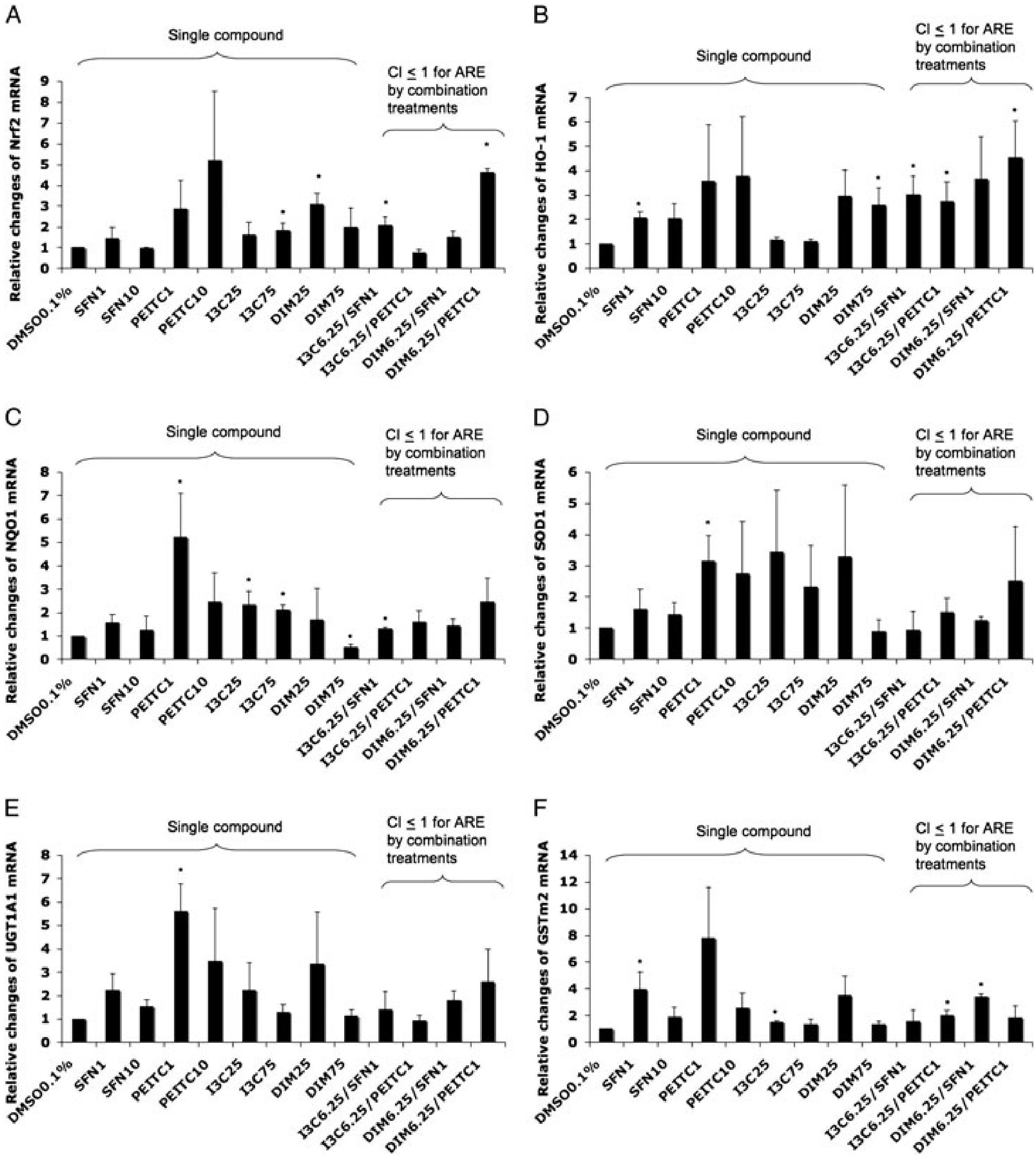

Figure 4. Real-time PCR (qPCR) results expressed in fold changes of mRNA over the control, using *GAPDH* as endogenous housekeeping gene. (A) Relative expression level of Nrf2 mRNA. (B) Relative expression level of HO-1 mRNA. (C) Relative expression level of NQO1 mRNA. (D) Relative expression level of SOD1 mRNA. (E) Relative expression level of UGT1A1 mRNA. (F) Relative expression level of GSTm2 mRNA. Results are expressed as mean ± SEM. The tested concentrations were in μM. *$p < 0.05$, compared with the 0.1% DMSO-treated control cells

by an increased consumption of fruits and vegetables containing rich sources of many phytochemicals [19]. The exact mechanisms by which phytochemicals could prevent diseases such as cancer are not clear, but would appear potentially to involve one of the signaling

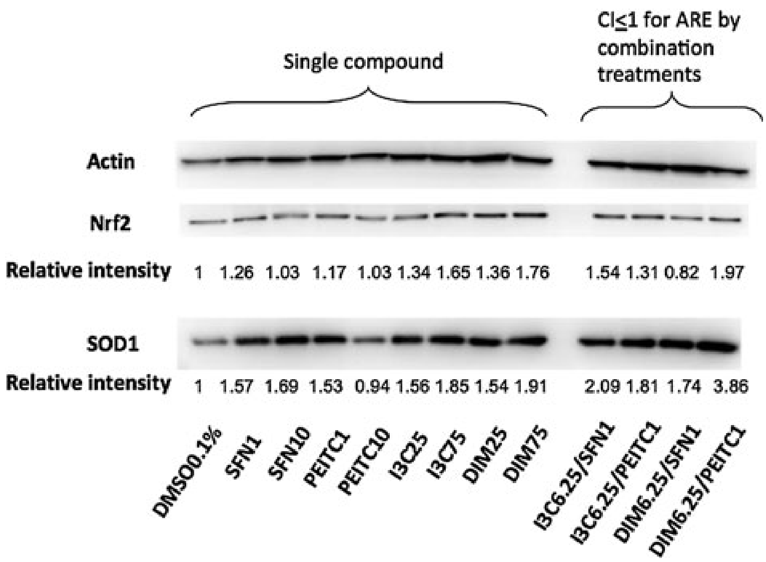

Figure 5. Effects of SFN, PEITC, I3C, DIM and their combinations on Nrf2 and SOD1 protein expression in HepG2-C8 cells by western blotting using actin as housekeeping protein. The combinations of low doses of indoles and ITCs were able to induce protein expression of Nrf2 and SOD1 and the synergism was evident for both Nrf2 and SOD1. The tested concentrations were in μM. Representative images of three independent experiments are shown

pathways, the Nrf2-ARE-mediated antioxidative stress pathway [8,12]. Similarly, the involvement of the Nrf2-ARE-mediated signaling by I3C and DIM remains unclear. Figure 6 shows the schematic diagram of the proposed mechanism by which Nrf2-ARE and its downstream targeting enzymes are induced by chemicals/phytochemicals, which has been proposed previously and reviewed [8,12]. In the current study, the transcriptional activation of Nrf2-ARE mediated gene expression was investigated, as well as the potential synergistic effects of the indoles and the ITC compounds.

As shown in Figure 3, SFN is a stronger inducer than PEITC in the ARE-luciferase transcription

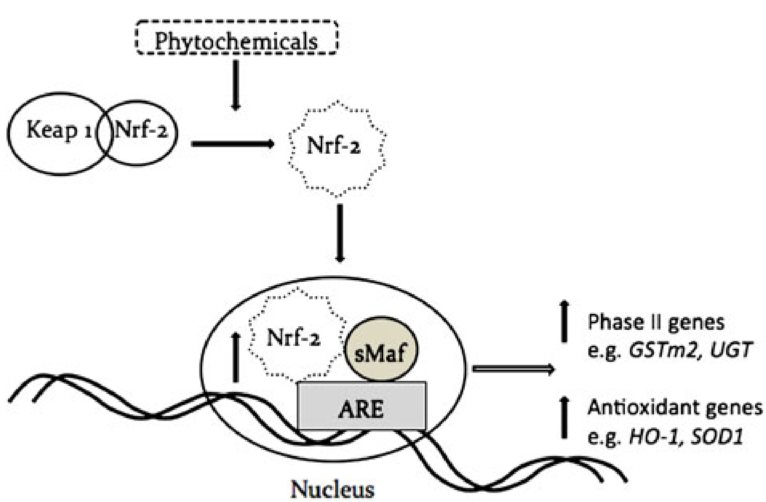

Figure 6. Schematic diagram of the proposed simplified pathway shows indole and isothiocyanate phytochemicals inducing Nrf2-ARE signaling through activation of the ARE and producing antioxidative and phase II detoxifying genes

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assay. However, in contrast, PEITC induced higher mRNA levels of endogenous Nrf2 and Nrf2-mediated genes than SFN (Figure 4). Previous reports show that HepG2 cells treated with PEITC [41] and SFN [42] have shown different time courses and concentration-dependent apoptosis. Since there is no existing report on the direct comparison of SFN and PEITC in the activities of Nrf2-ARE induction, time course studies were performed on the induction of ARE-luciferase activities by SFN and PEITC. As early as 6 h, 1 μM PEITC induced higher ARE activities than 1 μM SFN (data not shown). The slower inducing effect of SFN correlated with our previous report that SFN reached its peak induction at 18 h after treatment [35]. Moreover, there were also differences between SFN and PETIC with respect to treatment time for ARE activities (24 h, Figure 3), mRNA (6 h, Figure 4) and protein (24 h, Figure 5). It appears that the kinetic profiles for SFN and PEITC in inducing ARE, Nrf2 and Nrf2-mediated genes are quite different. These findings suggest that, in addition to the Nrf2-ARE mediated signaling pathway, other pathways such as the activation of the mitogen-activated protein kinases (MAPKs), could also be involved (reviewed in [21]).

In our current study, synergism was observed for different combinations between the indoles and the ITCs at some concentrations (Figure 3). In order to confirm the observations that the phytochemicals were promoting the induction of Nrf2, phase II DME and antioxidant genes, qPCR and western blotting were performed. Since preventing diseases including cancer initiation could be achieved by protecting cells and tissues against oxidative stress-mediated damage, an effective mechanism of defense against such damage could be by the induction of cellular phase II DME/detoxifying and antioxidant enzymes such as UGT, GST, NQO1, SOD1 and HO-1 [43]. The induction of these enzymes is mediated by the Nrf2-ARE signaling pathway. In this context, our current study quantifies the gene expression of *Nrf2*, *HO-1*, *SOD1*, *NQO1*, *UGT* and *GSTm2* and the induction of these genes is shown in Figure 4. The combination of 6.25 μM DIM plus 1 μM PEITC showed the most robust overall synergistic effect compared with the other treatments (Figure 4). Overall, it appears that 6.25 μM DIM with 1 μM PEITC would be the best combination under our experimental conditions, since synergistic induction was observed for all the genes studied (except *GSTm2*) and the fold induction was also relatively higher compared with the single agent treatment and the other combinations tested (Figure 4). In addition, Nrf2 and SOD1 proteins also showed potential synergism after 24 h of treatment with 6.5 μM DIM plus 1 μM PEITC (Figure 5).

It is highly likely that the metabolism in HepG2 cells would occur during the course of the studies, as reported by others [44–46], although our main focus is on the interactions between the indoles and ITCs with the Nrf2-ARE mediated phase II DME and antioxidant gene expression. It has been reported that I3C could be converted to DIM in culture medium and metabolized in breast cancer cells [44,45], the current study with equal doses of I3C and DIM at 25 μM and 75 μM did not produce equal ARE induction activities, indicating that the effects of I3C by itself might be active, although not as potent as DIM (Figure 3). This is an interesting observation indeed, since many more clinical studies have been performed using I3C than DIM [47]. In summary, it was shown that the indoles (I3C and DIM) and ITCs (PEITC and SFN) could induce *Nrf2* and its downstream genes synergistically at certain combinations. The indoles and ITCs are found abundantly in our daily consumed cruciferous vegetables. The potential of indoles to induce Nrf2-ARE-mediated phase II DME/antioxidant genes and the potential of synergism with ITCs has been suggested previously but has not been studied in detail. In the present study, it was found that both I3C and DIM could induce Nrf2-ARE-mediated luciferase reporter gene with DIM being the more potent inducer. Furthermore, both the indoles I3C and DIM displayed synergism with the ITCs, PEITC and SFN, in inducing Nrf2-ARE-mediated reporter gene as well as inducing endogenous phase II DME and antioxidant genes. The results of our current study would suggest potential synergistic cancer chemopreventive effects of indoles and ITCs *in vivo* as well as in humans.

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