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Minireview paper

Isothiocyanates: mechanism of cancer chemopreventive action

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Dietary and synthetic isothiocyanates have cancer chemopreventive activity. Dietary isothiocyanates are formed from glucosinolate precursors of ingested green vegetables. Isothiocyanates are absorbed across intestinal cell membranes by passive diffusion and bind reversibly to plasma protein thiols by thiocarbamoylation. Free isothiocyanate enters cells and is converted to the glutathione conjugate by glutathione S-transferases (GSTs). The glutathione conjugate is exported from cells by multidrug resistance proteins (MRPs), and metabolized in the mercapturic acid pathway to the corresponding mercapturic acid. The isothiocyanate is reformed by fragmentation of mercapturic acid pathway metabolites; it is inactivated by slow hydrolysis to the corresponding amine that is inactive in chemoprevention. Depletion of cellular glutathione and protein thiocarbamoylation activates signal transduction for cancer chemoprevention. Isothiocyanates inhibited and inactivated cytochrome P450 isoforms. They induced increased expression of GST, NADPH: quinone oxidoreductase, aldo–keto reductase and γ-glutamylcysteine synthetase. These responses were coordinated at the transcription level by nuclear factor-erythroid 2 p45-related factor-2 acting through the antioxidant/electrophile enhancer response element and stimulated by the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase-1 and c-Jun N-terminal kinase-1 (JNK1) pathway. Isothiocyanates also induced apoptosis of precancerous cells and tumor cells activated by caspase-8 and potentiated by JNK1. The chemopreventive activity of isothiocyanates is influenced by the isothiocyanate bioavailability—as is toxicity, GST polymorphism, protein thiocarbamoylation and probably also by MRP expression. These features of isothiocyanate metabolism and chemoprevention deserve further investigation.

Key words: Antioxidant response element, apoptosis, chemoprevention, isothiocyanate, multidrug resistance protein, nuclear factor-erythroid 2 p45-related factor-2.

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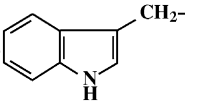
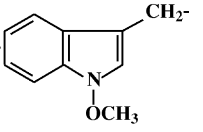
Introduction: dietary isothiocyanates

Epidemiological and pharmacological studies have indicated that dietary isothiocyanates are key mediators of the cancer chemopreventive activity associated with the consumption of cruciferous vegetables.[1,2] The major source of isothiocyanates absorbed form the diet is *Brassica* vegetables (broccoli, cauliflower, cabbage, watercress, radish and others). Isothiocyanates investigated for use as cancer chemopreventive agents are benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), allyl isothiocyanate (AITC), 4-methylsulphinylbutyl isothiocyanate (sulforaphane SFN) and others. They are formed from glucosinolate precursors, along with other products by the enzyme myrosinase (Table 1 and Figure 1). Myrosinase is segregated from glucosinolates in intact plant tissue. It gains access to glucosinolates after chopping of vegetables in culinary processing and chewing of raw vegetables; cooking inactivated myrosinase. Glucosinolates in cooked vegetables were converted to isothiocyanates by myrosinase of intestinal bacteria.[3] Optimum bioavailability of isothiocyanates was achieved by chewing of raw vegetables;[4] considerable loss of isothiocyanate occurred in cooking.[5] Isothiocyanates hydrolyzed spontaneously to corresponding amines, and reacted with cysteinyl thiols and lysyl and N-terminal amino groups to form dithiocarbamoyl and thiourea derivatives (Figure 2). The reaction of isothiocyanates with cellular glutathione (GSH) to form the conjugate RNHC(=S)–SG occurred non-enzymatically and enzymatically catalyzed by glutathione S-transferases (GSTs).[6,7]

The cancer chemopreventive activity of isothiocyanates has been linked to the inhibition of CYP isoforms that catalyze the activation of procarcinogens, induction of increased expression of enzymes involved in phase II detoxification of procarcinogens

Table 1. Glucosinolates and their hydrolysis products, isothiocyanates, thiocyanates and nitriles

Glucosinolate	Substituent R	R–N=C=S	R–S–C≡N	R–C≡N
Saturated aliphatic isothiocyanate				
Glucoiberin	3-methylsulfinylpropyl-$CH_3S{=}OCH_2CH_2CH_2$-	√		√
Glucoraphanin	4-methylsulfinylbutyl-$CH_3S{=}OCH_2CH_2CH_2CH_2$-	√		√
Glucoerysolin	4-methylsulfonylbutyl-$CH_3SO_2CH_2CH_2CH_2CH_2$-	√		√
Unsaturated aliphatic isothiocyanate				
Sinigrin	allyl-$CH_2{=}CH{-}CH_2$-	√		√
Gluconapin	butenyl-$CH_2{=}CH{-}CH_2CH_2$-	√		√
Aromatic isothiocyanate				
Glucotropaeolin	benzyl-$PhCH_2$-	√		√
Gluconasturtin	phenethyl-$PhCH_2CH_2$-	√		√
Indolyl isothiocyanate				
Glucobrassicin	3-indolylmethyl- 		√	√
Neoglucobrassicin	*N*-methoxy-3-indolylmethyl 		√	√

√ indicates a major degradation product.

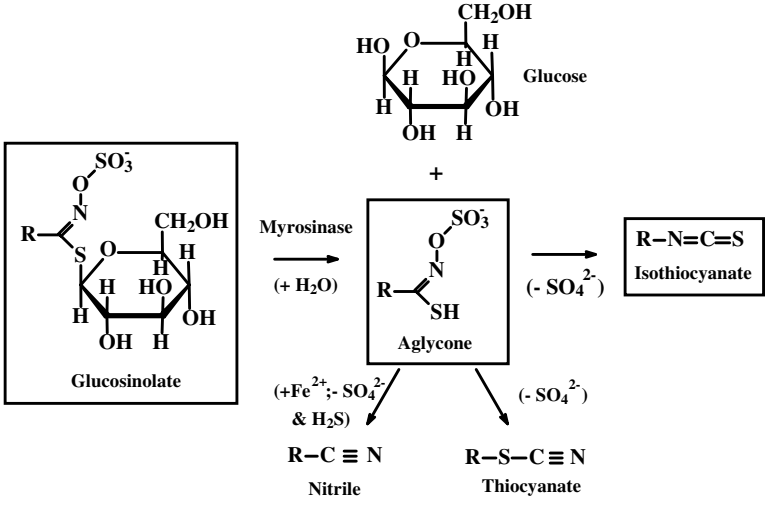


Figure 1. Formation of isothiocyanates, nitriles and thiocyanates from glucosinolates by myrosinase-catalyzed degradation.

and carcinogens [GSTs, NADPH: quinone oxidoreductase (QOR), aldo–keto reductase and γ-glutamylcysteine synthetase], and induction of apoptosis in pre-cancerous cells and tumor cells.

Effect of isothiocyanates on cytochrome P450 isoforms (CYPs)

Isothiocyanates inhibited rat and human CYP isoforms in liver microsomal preparations *in vitro*, including CYPs 1A1, 1A2, 2B1, 2B6, 2C9, 2D6, 2E1 and 3A4. These CYP isoforms were involved in the activation of many environmental carcinogens.[8–10] The inhibition of CYP by isothiocyanates is a competitive, non-competitive or other mechanism-based interaction, depending on the CYP isoform.[9,10] SFN inhibited the activities of CYP1A1 and 2B1/2 in rat hepatocytes and the expression of CYP3A4 in human hepatocytes.[11] It also inhibited carcinogen-induced DNA strand breaks in human hepatocytes expressing CYP1A2 and CYP2E1.[12] Clinical studies showed, however, that consumption of *Brassica*

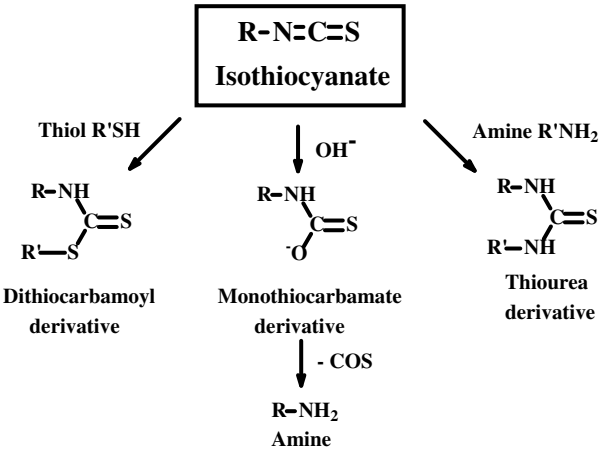


Figure 2. Reactions of isothiocyanates.

vegetables increased the activity of CYP1A2, as determined by caffeine metabolite ratios.[13,14] The expression of CYP1A1 and CYP1A2 was regulated at the transcriptional level by the antioxidant ARE found in the 5′-flanking region of these CYP genes.[15,16] The overall effect of chronic exposure to isothiocyanates on activities of CYP isoforms in human subjects is not clear.

GSTs in isothiocyanate metabolism and chemopreventive effects

Isothiocyanates entered cells by passive diffusion and were rapidly conjugated with GSH to form *S*-(*N*-alkylthiocarbamoyl)glutathione derivatives RNHC(=S)–SG catalyzed by GST isozymes.[6,7,17] This trapped isothiocyanates inside cells and the concentration of RNHC(=S)–SG maximized after about 30 min in cultured cells.[18–20] RNHC(=S)–SG was exported from cells by the GSH conjugate exporter multidrug resistance proteins (MRPs)[21] which decreased the cellular concentration RNHC(=S)–SG and hence also decreased the cellular concentration of GSH. Thereafter, isothiocyanate was bound reversibly to protein thiols (protein thiocarbamoylation).[18] The nadir of cellular GSH concentration and the zenith of protein thiocarbamoylation occurred after about 3 h in cultured cells. This coincided with the maximal induction of functional effects—induction of GST expression, activation of MAP kinases and commitment to apoptosis.[17,18,22,23] With median effective doses of isothiocyanates, very little formation of oxidized GSH (GSSG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine occurred in response to isothiocyanates.[18,24] The cellular concentration of GSH recovered from the minimum level to later restore a full complement of GSH.[18] Intrinsic oxygen free radical formation may not be critical to signal transduction of isothiocyanate responses.

SFN, BITC and PEITC induced the expression of GSTs in cultured cells. SFN induced the expression of GST-A in human colon adenocarcinoma cells.[25] BITC induced GSTP1 in rat liver epithelial cells.[23] In pre-clinical *in vivo* studies, aralkyl isothiocyanates and their cysteine conjugates increased GST activity in esophagus, small intestine and bladder.[26,27] Clinical studies indicated that dietary isothiocyanates induced increased activities of plasma GST-A and peripheral lymphocyte GST-M, and that this effect was more pronounced in female than male subjects.[28] Increased lymphocyte GST activity correlated with increased colonic mucosal GST activity.[29] Increased GST expression induced by isothiocyanates was coordinated through ARE in the 5′-flanking region of GST genes and nuclear factor-erythroid 2 p45-related factor-2 (Nrf2) and AP-1 transcription factors.[30,31] Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase-1 (MEKK1) by isothiocyanates and thereafter Jun N-terminal kinase-1 (JNK1) provided the signal transduction for these processes;[31–33] oxidative oligomerization of GST-P with subsequent dissociation of JNK from the GST-P/JNK inhibitory complex[34] may also be involved.

Increased expression of QOR and other enzymes

Non-toxic concentrations of isothiocyanates induced the expression of QOR, aldo–keto reductase 1C1 and γ-glutamylcysteine synthetase (heavy subunit) in human colonic carcinoma cells and murine hepatoma cells *in vitro*.[32,35,36] Increased expression of these enzymes by isothiocyanates was regulated by activation of MEKK1 and JNK, translocation of Nrf2 to the nucleus and dimerization with c-Jun, and binding of the Nrf2/c-Jun complex to ARE in the 5′-flanking region of their respective genes.[35,37–40]

Induction of apoptosis by isothiocyanates

Isothiocyanates inhibited the growth and induced apoptosis of tumor cells *in vitro*. The median growth inhibitory concentration GC_{50} values were in the range 0.8–8 μM. Cell growth was arrested in

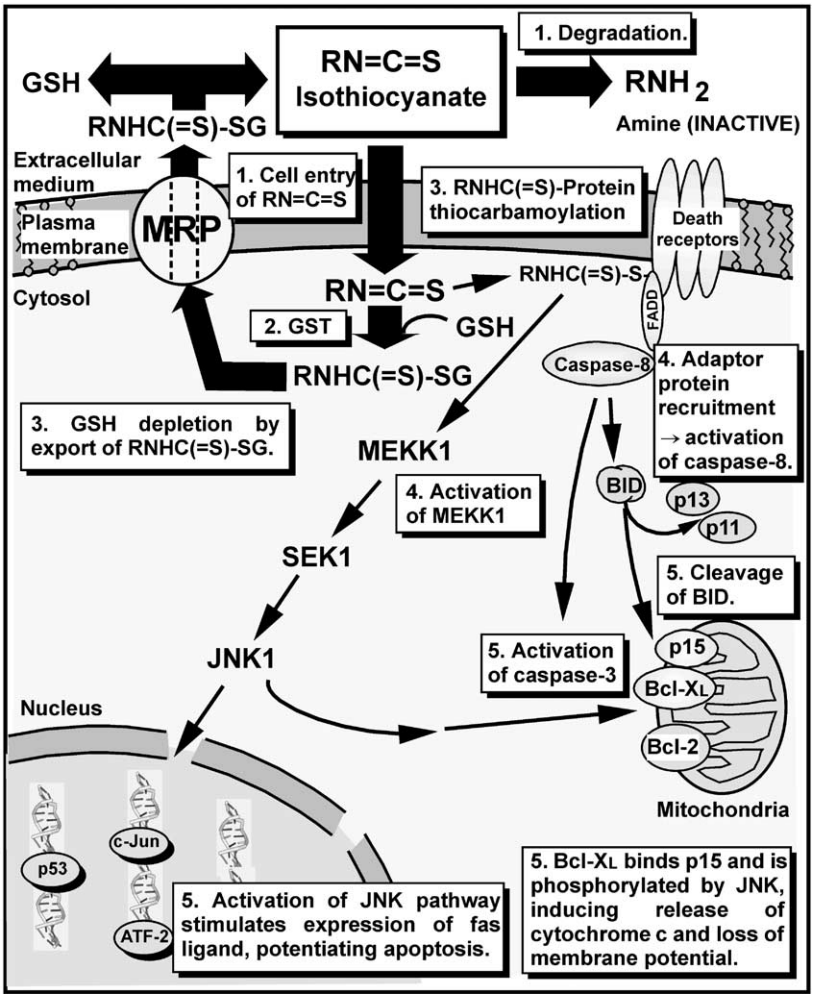


Figure 3. Induction of apoptosis by isothiocyanates.

G2/M and apoptosis was associated with activation of caspase-8, JNK1 and tyrosine phosphorylation.[17,18,22,33,41–44] The cytotoxicity of isothiocyanates was selective for tumor cells *in vitro*. The basis for this selectivity is unknown.

The mechanism of induction of apoptosis by isothiocyanates is not completely understood but the following sequence of events occurs (Figure 3):

(1) Entry of the isothiocyanate into cells by passive diffusion and concurrent slow inactivation by hydrolysis (Figure 2).
(2) Conversion of the isothiocyanate to RNHC(=S)–SG, catalyzed by GSTs.
(3) Depletion of cellular GSH by expulsion of RNHC(=S)–SG from cells by MRP. As the cellular concentration of GSH decreases, protein thiocarbamoylation by the isothiocyanate increases.
(4) When protein thiocarbamoylation maximizes, activation of the JNK and caspase-8 are maximal and commitment to apoptosis is achieved.
(5) Activation of caspase-3 and cleavage of cytosolic BID protein. Activation of the JNK pathway may potentiate apoptosis by inducing expression of the Fas ligand for the Fas death receptor.[45]
(6) Subsequent events lead to activation of DNA endonuclease, DNA fragmentation and cell death.[18]

Caspase-8 was activated by oligomerization at the cytosolic domain of a death receptor/FADD/procaspase-8 complex[46,47] and was probably a death ligand-independent response.[48] Caspase-8 and caspase-3 both cleaved BID protein to p15, p13 and p11 fragments.[49,50] p15 interacted with Bcl-x_L in mitochondria, leading to cytochrome *c* release and loss of mitochondrial membrane potential.[51] The tumor

suppressor protein p53 was involved in isothiocyanate-induced apoptosis,[52] but was not essential since apoptosis also occurred in p53 null cells.[17,42]

Involvement of apoptosis induced by isothiocyanates in cancer chemoprevention *in vivo* has been observed. Oral administration of the glucosinolate sinigrin that degraded to AITC induced apoptosis of aberrant colonic crypt cells induced by dimethylhydrazine induced in rats, indicative of a cancer chemopreventive effect.[53] Oral administration of PEITC to rats exposed to cigarette smoke-induced apoptosis of bronchial and alveolar epithelial cells and alveolar macrophages that had increased staining for proliferating cell nuclear antigen.[54] Apoptosis of cells exposed to the genotoxic agent may be viewed as a host protective response to pre-cancerous cells. Exposure to isothiocyanates potentiated this effect. Isothiocyanates also had antitumor activity *in vivo* and prevented the development of secondary tumors in experimental models of metastasis.[55,56]

Future directions

Association of chemopreventive activity of isothiocyanates with GST polymorphism

Decreased expression of GST is expected to lead to a longer biological half-life of isothiocyanates and confer increased cancer chemopreventive activity. Null genotypes for both GST-M1 and GST-T1 were associated with increased chemoprevention of lung cancer in current smokers and non-smokers with consumption of dietary isothiocyanates.[57–60] A homozygous deletion of GST-M1 gene gives rise to no GST-M1 activity in about 50% of the Caucasian population. The GST genotype appears to be an important determinant of the chemopreventive activity of isothiocyanates. This deserves further investigation.

Cell signaling induced by isothiocyanates

It is likely that the signal transduction activated by isothiocyanates inducing increased expression of phase II enzymes is also involved in the activation of apoptosis. The ARE is involved in phase I and phase II enzyme induction.[15,16] Signal transduction involves activation of MEKK1 and JNK, and binding of Nrf2/Jun protein complexes to ARE.[40,61] Activation of ERK2 may also be involved. Isothiocyanate-mediated thiocarbamoylation of Raf-1 was suggested as the initiation of the ERK2 pathway.[62] Activation of JNK1 was also associated with isothiocyanate-induced apoptosis.[22,44] It was proposed that low doses of isothiocyanate induced ARE-coordinated changes in gene expression and high doses of isothiocyanate activated apoptosis.[39] For aralkyl isothiocyanates such as BITC and PEITC, however, both changes in phase II gene expression and apoptosis occurred at similar concentrations of isothiocyanate.[17,32,35] During the period of induction of these responses by isothiocyanates, protein thiocarbamoylation was the major cellular metabolite of the isothiocyanate. Protein thiocarbamoylation was a reversible thiol modification which, similar to *S*-palmitoylation, may induce migration of proteins to the plasma membrane and re-orientation/re-folding of membrane and other proteins. This occurs temporarily.[18] I speculate that protein thiocarbamoylation may activate Raf-1, MEKK-1 and components of the death receptor/FADD/pro-caspase-8 complex, and thereby activate ERK2, JNK1 and caspase-8 in isothiocyanate-mediated responses. The role of protein thiocarbamoylation by isothiocyanates and activation of cell signaling requires further investigation.

GSH conjugate transporters and a role for isothiocyanates in therapy of multidrug resistance

Expulsion of the GSH conjugate RNHC(=S)–SG from cells decreased cellular GSH and was a critical feature for activation of cellular responses.[18,23] RNHC(=S)–SG expulsion is mediated by the MRPs. Indeed, SFN induced the expression of MRP-2.[63] MRPs have increased expression in some tumors and particularly in relapsed tumors with multidrug resistance.[64] High MRP expression is expected to produce a rapid expulsion of RNHC(=S)–SG from cells. Contrary to first thoughts, this may not necessarily decrease the chemopreventive activity of isothiocyanates but may rather increase the effect since depletion of GSH is critical to activating the cellular responses. The effect of MRP expression on cancer chemopreventive activity of isothiocyanates and the antitumor activity of isothiocyanates against tumors with MRP-associated multidrug resistance should be investigated.

Bioavailability of isothiocyanates

For the optimization of chemoprevention of cancer by isothiocyanates, it is important that the bioavailability of isothiocyanates from preparations for

prophylactic therapy and vegetables under conditions of routine culinary processing are characterized.[3,4] Isothiocyanates and glucosinolates are destroyed by prolonged cooking,[5] and it is likely that maximum dietary cancer chemoprevention is not currently being achieved in the general population—even those consuming *Brassica* vegetables frequently. Isothiocyanate bioavailability is also important in maintaining an acceptable safety margin in chemoprevention of cancer where increased exposure to isothiocyanates in prophylactic therapy and glucosinolate-enriched vegetables is now envisaged. Mercapturic acid derivatives of isothiocyanates fragment spontaneously to reform the isothiocyanate.[17,41] High concentrations of isothiocyanate and the corresponding amine in urine may cause the bladder hyperplasia and carcinoma found in rats given high doses of BITC and PEITC.[65] The pharmacokinetics and pharmacodynamics of isothiocyanates should be studied in human subjects to ensure that toxicity of isothiocyanates is avoided.

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