Chapter 9
The Chemopreventive Power of Isothiocyanates

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Abstract Isothiocyanates are derived from their naturally-occurring glucosinolate precursors, which are abundant in cruciferous vegetables. Numerous scientific studies beginning more than half a century ago have documented the chemoprotective activities of these compounds. Isothiocyanates have numerous protein targets through which they exert protection in the context of various diseases such as cancer, neurodegeneration, inflammatory disease, metabolic disease and infection. The major mechanisms by which the isothiocyanates confer protection involve induction of stress response pathways that restore the cellular redox and protein homeostasis, and contribute to resolution of inflammation. However, high concentrations of isothiocyanates cause cell cycle arrest and selectively kill cancer cells by inducing

This chapter is dedicated to Paul Talalay MD (1923–2019) whose vision, scientific rigour, and insightful mentoring have inspired the work and influenced the lives of generations of scientists. He was a pioneer in cancer chemoprevention, famously saying of the early days of the field 'no room was small enough to accommodate the few who were interested'. His leadership in quantitative discovery science culminated in the isolation from broccoli of the isothiocyanate sulforaphane as inducer of cytoprotective enzymes, leading to the exponential growth of research on sulforaphane worldwide and its current development for disease prevention in humans. Although we have lost our hero, the treasure of his legacy will always be kept.

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apoptosis, autophagy or necrosis. In this review, we present readers with a detailed overview of isothiocyanates functions and discuss their molecular targets and anti-neoplastic effects. Furthermore, we provide an up-to-date summary of the evidence on the chemoprotective activities of the most widely-studied isothiocyanates: sulforaphane, phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC).

Keywords Isothiocyanates · Cancer · Chemoprevention · Sulforaphane · PEITC · BITC

9.1 Introduction

Isothiocyanates (ITCs) are biologically active molecules which are derived from glucosinolate phytochemical precursors. Glucosinolates are *S*-β-thioglucoside *N*-hydroxysulfates (Fig. 9.1) that are abundant in cruciferous (Brassicacea) plants. Chemically, there are three different types of glucosinolates, according to the origin of their side chain: (1) aromatic (from Phe or Tyr); (2) aliphatic (from Leu, Ile, Met, or Val); and (3) indole (from Trp) (Fahey et al. 2001; Halkier and Gershenzon 2006). The same plants which contain glucosinolates also have β-thioglucosidase enzymes, known as myrosinases (EC 3.2.3.1), which, however, are physically separated from their glucosinolate substrates. Enzyme and substrate only come in contact when the integrity of the plant tissue is compromised, such as during injury or chewing. The myrosinase reaction results in rapid hydrolysis of the glucosinolates to give rise to a variety of reactive compounds (Fig. 9.1). ITCs represent one of the major types of products of the myrosinase reaction and contribute to most of the biological effects that have been associated with glucosinolates. Around 120 natural ITCs have been identified so far (Verkerk et al. 2009; Herr and Buchler 2010).

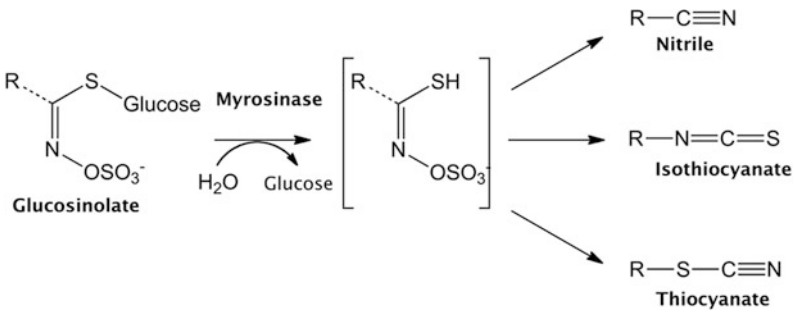


Fig. 9.1 The myrosinase reaction. Glucosinolates are hydrolyzed by β-thioglucosidases (myrosinases) to give unstable aglucones and liberate glucose. Depending on the reaction conditions, a variety of reactive products can be formed, the most common of which are nitriles, isothiocyanates and thiocyanates

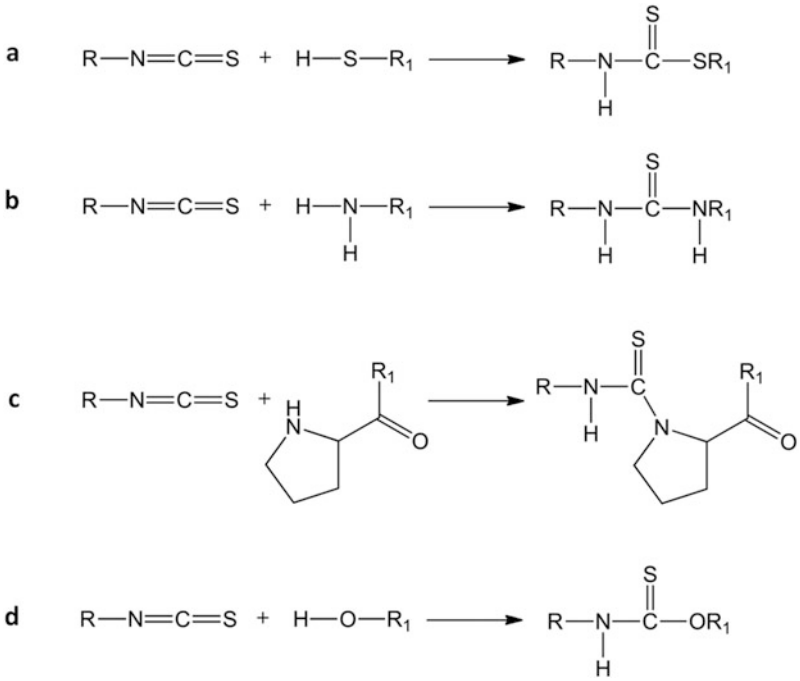


Fig. 9.2 Reactivity of isothiocyanates. The central carbon of the isothiocyante (—N=C=S) group is electrophilic and reacts readily with sulfur-, nitrogen-, and oxygen-centered nucleophiles. The most common reactions are: (**a**) conjugation with sulfhydryl groups, such as the sulfhydryl group of cysteine, (**b**) alkylation with α-amino groups in N-terminal residues and the ε-amino group of lysine, (**c**) reactions with the secondary amine in proline, and (**d**) reactions with hydroxyl group-containing residues, such as tyrosine

ITCs are characterized by high chemical reactivity due to the electrophilicity of the central carbon of the isothiocyanate (—N=C=S) group. The ITC group reacts readily with sulfur-, nitrogen-, and oxygen-centered nucleophiles (Fig. 9.2). Cysteine residues in proteins and glutathione (GSH) are the most common targets of ITCs, forming thiocarbamate products. The α-amino groups in N-terminal residues of proteins, the ε-amino groups of lysines, or even secondary amines, such as proline, can participate in alkylation reactions with ITCs, forming thiourea products. Finally, under certain although not physiological conditions, ITCs can also react with hydroxyl group-containing amino acid residues (e.g., tyrosine).

Natural ITCs or their synthetic analogs have been shown to prevent cancer development by limiting the exposure of cells to carcinogenic insults, thereby interfering with the initiation stage of carcinogenesis. The main mechanisms of early prevention include inhibition of intracellular activation of pro-carcinogens or acceleration of carcinogen detoxification. ITCs have been found to modulate transcript levels and inhibit phase I drug metabolizing enzymes, such as cytochrome P450 oxidases, involved in bioactivation of pro-carcinogens (Verkerk et al. 2009;

Herr and Buchler 2010). The potential and mechanisms of ITCs to induce cytoprotective enzymes are discussed in detail below, using sulforaphane as an example. Some of the mechanisms of cancer prevention by ITCs independent of their effects on carcinogen detoxification have been attributed at least in part to their cytotoxic properties. The current knowledge in this area will also be covered, emphasizing the molecular targets and signaling pathways contributing to ITCs toxicity towards cancer cells. In addition, ITCs have been reported to potently suppress the promotion and progression of carcinogenesis by affecting various signaling pathways related to inflammation (Heiss et al. 2001), angiogenesis (Xiao and Singh 2007; Bertl et al. 2006), autophagy (Powolny et al. 2011) metastasis formation (Wu et al. 2010), and dysregulation of gap junctional intercellular communications (Forster et al. 2014). Several reviews have discussed previously the various aspects of the mechanisms involved in the chemopreventive potential of ITCs (Antosiewicz et al. 2008; Cheung and Kong 2010; Fimognari et al. 2012; Jacob et al. 2011; Loo 2003; Prashar et al. 2012; Valgimigli and Iori 2009; Wu et al. 2009; Wu and Hua 2007; Zhang 2010; Zhang et al. 2005, 2006a).

9.2 The Diverse Family of Cytoprotective Proteins

A large family of proteins protects eukaryotic cells and organisms against the toxicities of electrophiles and oxidants which are the major causes of chronic degenerative diseases (Dinkova-Kostova and Talalay 2008, 2010). This family comprises enzymes that are involved in the metabolism and transport of a wide array of endo- and xenobiotics, as well as those that have direct and indirect antioxidant activities. Their functional diversity is truly extraordinary, and some examples are given in Table 9.1.

Notably, the distinction between *direct* and *indirect* antioxidant enzymes is not always straight-forward as many of these proteins perform both functions. For example, NQO1 is an *indirect* antioxidant enzyme because, by catalyzing the obligatory 2 electron-reduction of quinones, it prevents the formation of semiquinone radicals which, in the presence of oxygen, could lead to redox cycling, glutathione depletion, and oxidative stress (Dinkova-Kostova and Talalay 2010). NQO1 is also a *direct* antioxidant enzyme by virtue of its superoxide scavenging activity (Siegel et al. 2004). What is most important however is the fact that the inducibility and the enormous functional diversity of these cytoprotective enzymes underlie the capacity of the cell to mount a coordinate robust response to various conditions of stress, allowing adaptation and survival. It is thus not surprising that the genes coding for cytoprotective proteins share a common transcriptional regulation, with transcription factor Nrf2 (*N*F-E2 p45-*r*elated *F*actor *2*) being the master regulator of their expression (Motohashi and Yamamoto 2004). In addition to its direct influence on the transcription of cytoprotective genes, it is becoming increasingly clear that some of the protective effects of Nrf2 activation are mediated through cross-talks with other transcription factors, such as the aryl hydrocarbon receptor (AhR), nuclear factor κB (NF-κB), p53, and Notch1 (Wakabayashi et al. 2010).

Table 9.1 The diverse family of cytoprotective proteins

Protein function	Examples
Conjugation	Glutathione *S*-transferases (GSTs) UDP-glucuronosyltransferases (UGTs)
Export of xenobiotics and/or their metabolites	Solute carrier transporters ATP-binding cassette transporters
Synthesis, regeneration, utilization of glutathione	γ-Glutamate-cysteine ligase (γ-GCL) Glutathione reductase GSTs
Antioxidant enzymes	Heme oxygenase 1 (HO-1) NAD(P)H:Quinone oxidoreductase 1 (NQO1) GSTs
Synthesis of reducing equivalents	Glucose 6-phosphate dehydrogenase 6-Phosphogluconate dehydrogenase Malic enzyme 1 (ME1) Isocitrate dehydrogenase 1 (IDH1)
Anti-inflammatory enzymes	Leukotriene B_4 dehydrogenase
Prevention of damage by metal overload	Ferritin Metallothioneins
Repair and removal of misfolded or damaged proteins	Proteosomal subunits Proteins involved in autophagy

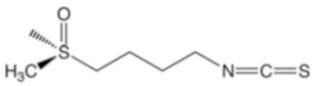


Fig. 9.3 Chemical structure of sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)butane]

9.3 Sulforaphane

9.3.1 Induction of Endogenous Cytoprotective Enzymes In Vitro

Sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)butane, Fig. 9.3] was isolated from extracts of broccoli (*Brassica oleracea*) as the principal inducer of the marker cytoprotective enzyme NQO1 using a highly quantitative bioassay in murine hepatoma Hepa1c1c7 cells (Zhang et al. 1992; Zhang and Tang 2007).

Over the years following this discovery, induction by sulforaphane of cytoprotective enzymes has been demonstrated in various cell culture and animal models by numerous independent research groups (Tables 9.2 and 9.3). In the Hepa1c1c7 cell line, sulforaphane treatment increased NQO1 and GST activities (Zhang et al. 1992; Gerhauser et al. 1997; Jiang et al. 2003; Matusheski et al. 2004; Anwar-Mohamed and El-Kadi 2009). Similar effects have been observed in rat bladder carcinoma NBT-II cells (Zhang et al. 2006b) and in murine NIH3T3 fibroblasts (Ernst et al. 2011). Exposure to sulforaphane in wild-type, but not

Table 9.2 Cytoprotective-inducing potential of sulforaphane in vitro

Organ	Cell line	Sulforaphane conc.	Treatment time	Cytoprotective enzymes induced	References
Liver	Hepa1c1c7	0.1–25 μM	24, 48 h	NQO1, GST	Zhang et al. (1992), Gerhauser et al. (1997), Jiang et al. (2003), Matusheski et al. (2004), Anwar-Mohamed and El-Kadi (2009)
	HepG2	5, 10, 12, 20, 25 μM	12, 24 h	NQO1, GST, UGT, HO-1, thioredoxin reductase 1, GSH	Jiang et al. (2003), Gan et al. (2010), Abdelhamid et al. (2010), Amara and El-Kadi (2011), Zhang et al. (2003), Bacon et al. (2003)
	Primary human hepatocytes	4–50 μM	48, 72 h	NQO1, GST	Gross-Steinmeyer et al. (2004), Maheo et al. (1997), Morel et al. (1997)
	Primary rat hepatocytes	10 μM	48, 72 h	GST	Maheo et al. (1997), Morel et al. (1997)
Breast	MCF7	25 μM	24 h	NQO1	Jiang et al. (2003)
	MCF10A	15 μM	24, 48 h	NQO1, AKRs, ALDH	Agyeman et al. (2012)
Prostate	LNCaP	0.1–25 μM	24, 48 h	NQO1, GST, HO-1	Jiang et al. (2003), Brooks and Paton (1999), Brooks et al. (2001), Clarke et al. (2011)
	LNCaPazaC	0.1–15 μM	48 h	NQO1, GST, γGCL	Brooks and Paton (1999)
	MDA PCa 2a	0.1–15 μM	48 h	NQO1, GST, γGCL	Brooks et al. (2001)
	MDA PCa 2b	0.1–15 μM	48 h	NQO1, GST, γGCL	Brooks et al. (2001)
	PC-3	0.1–15 μM	48 h	NQO1, GST, γGCL, HO-1	Brooks et al. (2001), Clarke et al. (2011)
	TSU-Pr1	0.1–15 μM	48 h	NQO1, GST, γGCL	Brooks et al. (2001)
	BPH1	15 μM	12 h	NQO1, HO-1	Clarke et al. (2011)
	PrEC	15 μM	12 h	NQO1, HO-1	Clarke et al. (2011)
Colon	HT-29	5–25 μM	2–24 h	NQO1, carbonyl reductase, γGCL, AKR1B1	Jiang et al. (2003), Ebert et al. (2010)
	Caco-2	1, 5, 10, 20, 50 μM	8, 24, 72 h	NQO1, GST, UGT, MRP2	Svehlikova et al. (2004), Jakubikova et al. (2005a), Traka et al. (2005)
Bladder	NBT-II	4, 8 μM	24 h	NQO1, GST	Zhang et al. (2006b)

Kidney	LLC-PK1	1, 3, 5 μM	24 h	NQO1, γGCL, GSH	Guerrero-Beltran et al. (2010)
Aorta	A10	0.5–5 μM	48 h	NQO1, GST, GSH, SOD, catalase, glutathione peroxidase, glutathione reductase	Zhu et al. (2008)
Spinal cord	Primary rat motor neurons	10 μM	48 h	NQO1, HO-1	Chang et al. (2010)
Brain	Primary murine cortical neurons	0.01–1 μM	24 h	NQO1, GST, glutathione reductase, thioredoxin reductase 1	Vauzour et al. (2010)
	Primary murine hippocampal neurons	0.5 μM	24 h	NQO1, HO-1, γGCL	Soane et al. (2010)
	Primary rat nigrostriatal cocultures	5 μM	48 h	NQO1	Siebert et al. (2009)
	Primary rat cortical astrocytes	5, 10 μM	24, 48 h	NQO1, HO-1, γGCL, thioredoxin reductase	Danilov et al. (2009), Bergstrom et al. (2011)
	SK-N-SH	0.5–5 μM	24 h	NQO1, GSH	Mas et al. (2012)
Retina	ARPE-19	0.625, 2.5 μM	24 h	NQO1, glucose-6-phosphate dehydrogenase, glutathione reductase	Gao et al. (2001)
Lung	Primary human bronchial epithelial cells	1 μM	24, 48 h	NQO1	Tan et al. (2010)
	HBEC	1 μM	24, 48 h	NQO1	Tan et al. (2010)
	BEAS-2B	5 μM	12, 24 h	NQO1, GST, HO-1, γGCL	Starrett and Blake (2011), Ritz et al. (2007)
Skin	Normal human keratinocytes	2.5, 5, 25 μM	24 h	NQO1, γGCL	Marrot et al. (2008)
	Normal human melanocytes	1, 5, 10 μM	24 h	NQO1, HO-1, γGCL	Marrot et al. (2008)
	HaCaT human keratinocytes	1–5 μM	24, 48 h	NQO1, GSH, HO-1, γGCL	Dinkova-Kostova et al. (2006), Wagner et al. (2010), Zhu and Bowden (2004)
	PE murine keratinocytes	0.2–5 μM	24, 48 h	NQO1, GSH	Dinkova-Kostova et al. (2006)

(continued)

Table 9.2 (continued)

Organ	Cell line	Sulforaphane conc.	Treatment time	Cytoprotective enzymes induced	References
Blood	CD34-derived human dendritic cells	2 μM	6, 24 h	NQO1, HO-1	Ade et al. (2009)
	THP-1 myeloid cells	2 μM	6, 24 h	NQO1, HO-1	Ade et al. (2009)
	Ramos 2G6 human B lymphocytes	5–20 μM	16 h	NQO1, GST	Wan and Diaz-Sanchez (2006)
	Human PBMC	5–20 μM	16 h	NQO1, GST	Wan and Diaz-Sanchez (2006)
Fibroblasts	Mouse embryonic fibroblast (MEFs)	3, 4, 5, 8 μM	24 h	NQO1, GST, γGCL, GSH	Zhang et al. (2006b), Nioi et al. (2003), Higgins and Hayes (2011)
	NIH3T3 fibroblasts	5, 10 μM	24 h	NQO1, γGCL, HO-1	Ernst et al. (2011)

Table 9.3 Cytoprotective-inducing potential of sulforaphane in vivo

Organ	Species	Sulforaphane dose and route of administration	Treatment duration	Cytoprotective enzymes induced	References
Liver	Mouse	15 μmol/day, *p.o.*	5 days	NQO1, GST	Zhang et al. (1992), Hu et al. (2006a)
		90 mg/kg, *p.o.*	Single dose	Phase 2 and 3 drug metabolizing enzymes, heat shock proteins, proteasomal subunits	
	Rat	40, 200, 500, or 1000 μmol/kg, *p.o.*	5 days	NQO1, GST	Matusheski and Jeffery (2001), Munday and Munday (2004)
Forestomach	Mouse	15 μmol/day, *p.o.*	5 days	NQO1, GST	Zhang et al. (1992)
Stomach	Mouse	15 μmol/day, *p.o.*	5 days	NQO1, GST	Zhang et al. (1992)
	Rat	40, 80, or 160 μmol/kg, *p.o.* As broccoli extract	14 days	NQO1, GST	Zhang et al. (2006b)
		40, 80, or 160 μmol/kg in diet as broccoli extract	14 days		
Small intestine	Mouse	15 μmol/day, *p.o.*	5 days	NQO1, GST	Zhang et al. (1992), McMahon et al. (2001), Thimmulappa et al. (2002)
		3 μmol/g of diet	14 days	NQO1, GST	
		9 μmol/day, *p.o.*	7 days	NQO1, GST, γGCL, UGT, epoxide hydrolase, glutathione peroxidase, glutathione reductase, ferritin, haptaglobin, NADPH regenerating enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme)	

(continued)

Table 9.3 (continued)

Organ	Species	Sulforaphane dose and route of administration	Treatment duration	Cytoprotective enzymes induced	References
Colon	Rat	40, 200, 500, or 1000 μmol/kg, *p.o.*	5 days	NQO1, GST	Matusheski and Jeffery (2001), Munday and Munday (2004)
Pancreas	Rat	40, 200, 500, or 1000 μmol/kg, *p.o.*	5 days	NQO1, GST	Matusheski and Jeffery (2001), Munday and Munday (2004)
Lung	Mouse	15 μmol/day, *p.o.*	5 days	NQO1, GST	Zhang et al. (1992)
Mammary gland	Mouse	3 mg/mouse, *p.o.*	4 days	NQO1, GST	Gerhauser et al. (1997)
	Rat	150 μmol, *p.o.*	Single dose	NQO1, HO-1	Cornblatt et al. (2007)
Retina	Mouse	0.5 mg/day, *i.p.*	3 days	Thioredoxin, thioredoxin reductase	Kong et al. (2007), Tanito et al. (2005)
Brain	Mouse	50 mg/kg, *i.p.*	16 h	NQO1, HO-1	Innamorato et al. (2008), Jazwa et al. (2011)
	Rat	5 mg/kg, *i.p.*	Single dose	NQO1, GST., SOD, catalase, HO-1	Zhao et al. (2007a), Hong et al. (2010), Ping et al. (2010), Chen et al. (2011)
Spinal cord	Rat	5 mg/kg, *i.p.*	Single dose	NQO1, HO-1, γGCL	Wang et al. (2012b)
Sciatic nerve	Mouse	0.5 or 1 mg/kg	14 days	NQO1, HO-1	Negi et al. (2011)
Skin	Mouse	1 μmol/day, topically	3 days	NQO1, K16, K17	Kerns et al. (2007), Talalay et al. (2007)

(continued)

Table 9.3 (continued)

Organ	Species	Sulforaphane dose and route of administration	Treatment duration	Cytoprotective enzymes induced	References
Bladder	Rat	40 μmol/kg, *p.o.*	5 days	NQO1, GST	Zhang et al. (2006b), Munday and Munday (2004), Munday et al. (2008)
		160 μmol/kg in diet as broccoli extract	6 and 12 weeks		
		40, 80, or 160 μmol/kg, *p.o.*, as broccoli extract	14 days		
		40, 80, or 160 μmol/kg in diet as broccoli extract	14 days		

Nrf2-knockout primary mouse embryonic fibroblasts (MEFs), caused an induction of 2- to 10-fold in the levels of mRNA for γ-glutamate-cysteine ligase (γ-GCL) catalytic (GCLC) and modifier (GCLM) subunits, GSTs and NQO1, and increased the levels of total GSH by 1.5- to 1.9-fold (Nioi et al. 2003; Higgins and Hayes 2011). In porcine renal epithelial cells (LLC-PK1), sulforaphane induced NQO1 and γGCL, increased the levels of GSH, and protected against cisplatin-mediated oxidative stress, mitochondrial membrane depolarization and cell death (Guerrero-Beltran et al. 2010). Furthermore, in isolated renal mitochondria from Wistar rats that had been treated with cisplatin, two intravenous injections of sulforaphane (the first one 24 h before and the second one 24 after cisplatin treatment) prevented the cisplatin-induced increase in reactive oxygen species and depletion of GSH, and restored the ATP content and oxygen consumption (Guerrero-Beltran et al. 2010). Similar protection was also observed in liver of cisplatin-treated animals (Gaona-Gaona et al. 2011). Exposure of rat aortic smooth muscle A10 cells to sulforaphane resulted in the induction of a number of cytoprotective enzymes in both whole-cell lysates as well as in mitochondrial fractions, including NQO1, superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, GST, increased the levels of GSH, and protected against the toxicities of oxidants and electrophiles, such as superoxide, H_2O_2, peroxynitrite, 4-hydroxy-2-nonenal, and acrolein (Zhu et al. 2008).

In motor neurons grown in organotypic cultures of rat spinal cord, sulforaphane induced NQO1 and heme oxygenase 1 (HO-1), and protected against glutamate-mediated excitotoxicity (Chang et al. 2010). Protection by sulforaphane against 5-*S*-cysteinyl-dopamine-induced neuronal injury was observed in cultures of primary murine cortical neurons and shown to correlate with increased expression and activity of the M-class (M1, M3 and M5) GSTs, glutathione reductase, thioredoxin

reductase and NQO1 (Vauzour et al. 2010). In primary murine hippocampal neurons exposed to hemin or to the combination of oxygen and glucose deprivation, treatment with sulforaphane during the reoxygenation phase induced NQO1, HO-1 and GCLM, and protected against cell death (Soane et al. 2010). Dopaminergic neurons that were isolated from Sprague-Dawley rats and grown in organotypic nigrostriatal cocultures were protected against the toxicity of 6-hydroxydopamine by prior treatment with sulforaphane, and the observed protection was attributed to the increase in antioxidant capacity (Siebert et al. 2009). When primary cultures of rat cortical astrocytes were exposed to sulforaphane either 48 h prior to, or for 48 h after, a 4-h period of oxygen and glucose deprivation, both pre- and post-treatment was protective against oxidative stress (assessed by immunostaining for 8-hydroxy-2-deoxyguanosine) and cell death, with the concomitant induction of the levels of mRNA, protein, and enzyme activity of NQO1 (Danilov et al. 2009). In a similar cell culture system, sulforaphane exposure for 4 h induced the levels of mRNA for NQO1 and HO-1 (Bergstrom et al. 2011). Repeated sulforaphane administration resulted in an accumulation of mRNA and protein levels of NQO1 and was protective against oxidative damage. Similar effects of sulforaphane were also observed in human adult retinal pigment epithelial cells (ARPE-19), keratinocytes (HaCaT), and murine leukemia (L1210) cells (Gao et al. 2001).

In primary normal human bronchial epithelial cells as well as in the immortalized human bronchial epithelial cell line HBEC, sulforaphane caused a robust upregulation of NQO1 mRNA and protein levels (Tan et al. 2010). Induction of NQO1 by sulforaphane was also observed in primary cultures of human hepatocytes (Gross-Steinmeyer et al. 2004). In the Caco-2 human colon cancer cell line, NQO1, multidrug resistance-associated protein 2 (MRP2), GSTA1 and UDP-glucuronosyltransferase were elevated upon sulforaphane treatment (Svehlikova et al. 2004; Jakubikova et al. 2005a). In the human hepatoma cell line HepG2, sulforaphane treatment also increased the transcription of the endogenous NQO1 (Gan et al. 2010; Abdelhamid et al. 2010; Amara and El-Kadi 2011), thioredoxin reductase 1 (Zhang et al. 2003), and HO-1 (Gan et al. 2010). Moreover, in combination with selenium, sulforaphane treatment resulted in protection against paraquat-induced cell death (Zhang et al. 2003). In primary human and rat hepatocytes, sulforaphane induced the transcription of GSTA1/2 mRNA (Maheo et al. 1997; Morel et al. 1997). In HepG2 cells and in primary human hepatocytes, treatment with sulforaphane inhibited the formation of PhiP-DNA adducts; the protective effect correlated with transcriptional upregulation of UDP-glucuronosyltransferase and GSTA1 (Bacon et al. 2003). In the human dopaminergic neuroblastoma SK-N-SH cell line, sulforaphane induced NQO1 enzyme activity and increased the levels of glutathione (Mas et al. 2012). Induction of NQO1 was also observed in a number of human prostate cancer cell lines (Jiang et al. 2003; Brooks and Paton 1999; Brooks et al. 2001). The levels of GSH and GSH-related enzymes, such as GCLC and GSTs, were also upregulated (Brooks and Paton 1999). In the human BEAS-2B epithelial cell line, sulforaphane increased the expression of NQO1, HO-1, and GCLM (Starrett and Blake 2011). Sulforaphane also increased the levels of mRNA for HO-1 and NQO1 in normal (PrEC), benign hyperplastic (BPH1) and cancerous (LNCaP and PC3) human prostate epithelial

cells (Clarke et al. 2011), and the mRNA for carbonyl reductase 3, a member of the short-chain dehydrogenase/reductase superfamily, in HT-29 colon cancer cells (Ebert et al. 2010). Upregulation of NQO1 was observed when cultured normal human keratinocytes and melanocytes were exposed to sulforaphane (Marrot et al. 2008). In murine and human (HaCaT) keratinocytes, the enzyme activity of NQO1, the GSH content (Zhu et al. 2004; Dinkova-Kostova et al. 2006) and the mRNA and protein levels for NQO1, HO-1 and γ-GCL (Wagner et al. 2010) were all upregulated by exposure to sulforaphane. NQO1 and HO-1 were also induced by sulforaphane in human CD34-derived dendritic cells isolated from umbilical cord blood, and in the THP-1 myeloid cell line (Ade et al. 2009). Sulforaphane treatment also caused increased gene expression of *NQO1*, *GSTM1* and *GSTP1* in cultured Ramos 2G6 human B lymphocytes and PBMCs isolated from blood (Wan and Diaz-Sanchez 2006), as well as in the airway epithelial cell line BEAS-2B (Ritz et al. 2007). In the estrogen receptor negative human breast epithelial MCF10A cell line, sulforaphane exposure led to a profound upregulation of the aldo-keto reductase family members AKR1B10, AKR1C1, AKR1C2 and AKR1C3, the aldehyde dehydrogenase 3 family member ALDH3A1, and of NQO1, as revealed by the use of both microarray and stable isotopic labeling with amino acids in culture (SILAC) approaches (Agyeman et al. 2012).

9.3.2 *Activation of the Antioxidant/Electrophile Responsive Element in Reporter Systems*

The upstream regulatory regions of the genes coding for cytoprotective proteins contain single or multiple copies of the antioxidant/electrophile response element (ARE/EpRE, consensus sequence: 5′-A/GTGAC/GNNNGCA/G-3′) (Rushmore and Pickett 1990; Friling et al. 1990; Hayes et al. 2010). Activation of gene expression through the ARE requires binding of transcription factor Nrf2 as a heterodimer with a small Maf protein (Motohashi and Yamamoto 2004). Thus, in addition to the effects on endogenous gene expression, ARE- and Nrf2-dependent induction by sulforaphane has been demonstrated in a number of reporter systems. In the human hepatoma cell line HepG2 stably transfected with the chloramphenicol acetyltransferase (CAT) reporter gene under the transcriptional control of the rat GSTYa promoter, treatment with sulforaphane caused a dose-dependent induction of the reporter gene (Fei et al. 1996). Similarly, a second reporter HepG2 cell line that was developed by a stable transfection with the gene encoding green fluorescent protein (GFP) under the transcriptional control of the thymidine kinase (TK) promoter adjacent to the ARE, also showed an increase in fluorescence upon sulforaphane treatment (Zhu and Fahl 2000). When HepG2 cells were transiently transfected with a CAT reporter under the control of the 5′-regulatory region of the rat NQO1 gene and then exposed to sulforaphane, enhanced CAT expression was also observed (Gerhauser et al. 1997). Sulforaphane caused induction of the

ARE-luciferase reporter in the stably transfected MCF7-derived AREc32 human breast cancer cell line, which contains a luciferase reporter construct controlled by eight copies of the ARE that is present in both rat *GSTA2* and mouse *gsta1 genes* (Wang et al. 2006, 2010; Dinkova-Kostova and Wang 2011). The pARE-TI-luciferase reporter in the stably transfected HepG2-ARE-C8 cell line was also induced by sulforaphane (Saw et al. 2011). Upregulation of the luciferase reporter was observed in the reporter cell line EpRE(mGST-Ya)-LUX, which is a Hepa1c1c7 cell line that contains the ARE from the promoter region of the murine *gstya* gene (Vermeulen et al. 2009). Sulforaphane was also shown to activate a reporter in which the Neh2 domain of Nrf2 that is responsible for binding to its negative regulator, Kelch-like ECH-associated protein 1 (Keap1) (Itoh et al. 1999), was fused to firefly luciferase (Neh2-luciferase) allowing the direct monitoring of the response to inducers based on the time course of reporter activation (Smirnova et al. 2011).

9.3.3 Cytoprotective Effects of Sulforaphane In Vivo

Induction of cytoprotective enzymes by sulforaphane also occurs in vivo (Table 9.3). Sulforaphane administered to mice daily, *p.o.*, at a dose of 15 μmol/day, for 5 days, resulted in induction of NQO1 and GST activities in liver, forestomach, glandular stomach, small intestine, and lung (Zhang et al. 1992). Induction of both NQO1 and GST occurred in mammary glands of mice that had been given 4 daily doses of 3 mg of sulforaphane per animal, *p.o.* (Gerhauser et al. 1997). In liver, colon, and pancreas of rats given either 200, 500, or 1000 μmol/kg (Matusheski and Jeffery 2001) or 40 μmol/kg of sulforaphane, *p.o.* for 5 days, NQO1 and GST activities were also upregulated (Munday and Munday 2004). Especially high was the magnitude of induction in bladder (Zhang et al. 2006b; Munday and Munday 2004; Munday et al. 2008). Feeding sulforaphane (3 μmol/g of diet) for 14 days induced the activities of NQO1 and GST in the small intestine in wild-type mice (McMahon et al. 2001). In contrast, an identical treatment had no effect in Nrf2-knockout animals (McMahon et al. 2001). Topical application of sulforaphane to the mouse skin induced the gene expression of keratins 16 (K16) and 17 (K17) in the basal layer of the epidermis (Kerns et al. 2007). K17 is homologous and functionally redundant with K14, and the sulforaphane-dependent induction of K17 was evaluated as a potential strategy for reducing skin blistering in K14-knockout mice, a model for the human skin blistering disease epidermolysis bullosa simplex (EBS). Systemic (*i.p.*) administration of 5 μmol of sulforaphane to a pregnant mouse every other day in the week before delivery followed by topical applications of 1 μmol of sulforaphane to the newborn pups on the day of birth, day 1 and day 3 after birth, restored the skin integrity in these animals (Kerns et al. 2007). A subsequent study revealed that the induction of K17 is independent of Nrf2 activity and parallels the decrease in glutathione levels that occur after topical administration of sulforaphane (Kerns et al. 2010).

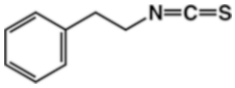
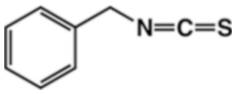
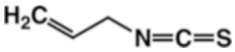
A pharmacokinetics/pharmacodynamics preclinical study for breast cancer prevention by sulforaphane was conducted in Sprague-Dawley rats (Cornblatt et al. 2007). It was found that orally administered sulforaphane reaches the mammary gland and increases the levels of cytoprotective enzymes in this tissue. The levels of dithiocarbamates (sulforaphane and its glutathione-derived conjugates) peaked at 1 h after oral administration of 150 μmol sulforaphane, reaching concentrations of 60 μM and 18.8 pmol/mg tissue in plasma and mammary gland, respectively. After 1 h, the plasma concentration of dithiocarbamates declined rapidly and exhibited a minor second peak of 22 μM at 12 h. The mRNA levels for NQO1 in the mammary gland were significantly induced as early as 2 h after dosing, and maximally elevated at 12 h. The NQO1 enzymatic activity in the mammary gland was also increased, peaking at 24 h after dosing. A biphasic pattern of HO-1 transcript induction was observed, with an initial peak at 2 h followed by a second peak at 12 h, indicating a more complex mode of regulation.

Microarray analyses in cells and tissues isolated from mice, rats and humans have further confirmed and expanded the list of transcriptional targets of sulforaphane (Agyeman et al. 2012; Thimmulappa et al. 2002; Hu et al. 2004, 2006a; Traka et al. 2005, 2008). The most prominent changes are in genes encoding proteins that are involved in xenobiotic metabolism, glutathione homeostasis, carbohydrate metabolism, and NADH/NADPH regeneration, and are thus tightly linked to cellular defense mechanisms, inhibition of proliferation, and induction of differentiation. The multitude of effects of sulforaphane on such fundamental cellular processes has led to numerous investigations evaluating the ability of this isothiocyanate to protect against the development of chronic degenerative diseases. Indeed, protection by sulforaphane has been demonstrated in animal models of carcinogenesis (Zhang et al. 1994; Chung et al. 2000; Fahey et al. 2002; Conaway et al. 2005; Kuroiwa et al. 2006; Myzak et al. 2006; Hu et al. 2006b; Gills et al. 2006; Xu et al. 2006; Singh et al. 2009), cardiovascular disease (Piao et al. 2010), diabetes (Zheng et al. 2011; Negi et al. 2011), neurotoxicity (Innamorato et al. 2008; Toyama et al. 2011), neurodegeneration (Kong et al. 2007; Rojo et al. 2010; Innamorato et al. 2010; Jazwa et al. 2011), and neuronal tissue injury (Tanito et al. 2005; Zhao et al. 2005, 2007a, b; Dash et al. 2009; Hong et al. 2010; Ping et al. 2010; Chen et al. 2011; Mao et al. 2011; Wang et al. 2012a). Notably, all of these disease models have both oxidative stress and inflammatory components underlying their pathogenesis. The protective effects of sulforaphane in animal models are being reflected in multiple human studies, in which various broccoli preparations or dietary supplements have been used as delivery vehicles for sulforaphane. The published studies have been summarized in a recent review (Dinkova-Kostova et al. 2017), and there are 20 ongoing clinical trials. In addition, a stabilized version of suforaphane encapsulated in cyclodextrin (SFX-01) is currently in two clinical trials, in patients with subarachnoid haemorrhage (NCT02614742) and metastatic breast cancer (NCT02970682) (Cuadrado et al. 2019).

9.3.4 *Long Lasting Indirect Antioxidant Properties Via Induction of Cytoprotective Enzymes*

The ability to upregulate the expression of a plethora of cytoprotective genes and to inhibit pro-inflammatory responses makes sulforaphane a particularly efficient, albeit indirect antioxidant. The "ultimate antioxidants," namely, the cytoprotective enzymes, act catalytically, are not consumed in the course of their antioxidant functions, have relatively long (usually several days) half-lives, and catalyze a wide variety of chemical reactions, such that their concerted actions protect cells and organisms and allows their adaptation to conditions of stress. A study using human adult retinal pigment epithelial cells (ARPE-19) as a model demonstrated that induction of cytoprotective enzymes by sulforaphane is a powerful strategy for enhancing the cellular antioxidant defense (Gao et al. 2001). Furthermore, such intervention provides efficient protection against chemically-induced oxidative stress produced by oxidants of several different types, such as the redox cycling agent menadione, the water-soluble peroxide *tert*-butyl hydroperoxide, the genotoxic alkenal 4-hydroxynonenal, and the highly damaging product of the reaction of superoxide with nitric oxide, peroxynitrite. Unlike the short-lived effects of direct antioxidants, protection against menadione toxicity at the end of a 24-h treatment with sulforaphane is prolonged and maintained for several days. Importantly, the duration of sulforaphane-mediated resistance to menadione paralleled the time period of increased cytoprotective enzyme activities: NQO1, glucose-6-phosphate dehydrogenase, and glutathione reductase in cells treated with sulforaphane continued to rise for 48 h after removal of sulforaphane from the medium and remained high during the ensuing 48–72 h. The levels of GSH after 24-h exposure to sulforaphane were increased by 50%, remained at this level for another 24 h, and then declined to control cell levels in the subsequent 96 h. In primary cultures of rat cortical astrocytes, sulforaphane exposure for 4 h induced the levels of mRNA for NQO1 and HO-1 (Bergstrom et al. 2011). These levels remained high for 24 h, and the corresponding protein levels were increased for more than 48 h. The long-lasting effects of sulforaphane treatment on induction of NQO1 were also observed in several different human prostate cancer cell lines (Jiang et al. 2003; Brooks and Paton 1999; Brooks et al. 2001). Transcriptional induction by sulforaphane was transient: it was evident at 4 h after exposure, reached a peak at 8 h, and returned to basal levels by 12 h. However, the enzyme activity remained elevated for up to 5 days after treatment. The levels of glutathione and glutathione-related enzymes, such as GCLC and GSTs, were also upregulated (Brooks and Paton 1999). Thus, even a transient exposure to sulforaphane causes an elevation of endogenous antioxidant systems, which by virtue of their long half-lives ultimately result in long-lasting protection. Moreover, protection against oxidative stress is quantitatively related to the indirect antioxidant action of sulforaphane, which is the result from induction of cytoprotective enzymes.

Table 9.4 Structure, chemical names and dietary sources of selected isothiocyanates (Cheung and Kong 2010; Prashar et al. 2012)

Structure	Chemical name/ Glucosinolate precursor	Origin
	2-Phenethyl-ITC (PEITC) 2-Isothiocyanatoethyl benzene/Gluconasturtiin	Watercress, radishes, turnips
	Benzyl-ITC (BITC) Isothiocyanatomethyl benzene/Glucotropaeolin	Lepidium cress, cabbage, papaya
	Allyl ITC (AITC) 3-Isothiocyanatoprop-1-ene/Sinigrin	Mustards, horseradish, wasabi, cabbage, brussels sprouts, kale, cauliflower

9.4 Phenethyl Isothiocyanate, Benzyl Isothiocyanate, Allyl Isothiocyanate

Phenethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC) and allyl isothiocyanate (AITC) occurring commonly in the human diet (Table 9.4) have been widely investigated with respect to their cancer chemopreventive properties.

AITC, also known as mustard oil, is responsible for the pungent taste of several cruciferous vegetables. This is due to its effects on the transient receptor potential A1 channel in sensory neurons and plays a role in plant defense against herbivores (Zhang 2010). Moreover AITC, as well as sulforaphane, PEITC and BITC, manifest antimicrobial activity against a wide spectrum of pathogens (Zhang 2010; Fahey et al. 2002; Navarro et al. 2011; Haristoy et al. 2005; Johansson et al. 2008; Yanaka et al. 2009). Many studies have presented AITC, sulforaphane, PEITC and BITC as promising cancer preventive phytochemicals, with anti-cancer activity in both cancer cells and animal models (Zhang 2010; Navarro et al. 2011). Many examples of comparison studies performed with use of aliphatic AITC and sulforaphane and aromatic PEITC and BITC have enabled to draw conclusions about their structure-activity relationships amongst compounds belonging to the ITC family (Prashar et al. 2012). Other ITCs, like sulforaphane, are able to induce cytoprotective enzymes responsible for detoxification and augmentation of anti-oxidant defense, as outlined above. The potency of ITCs to induce apoptosis or inhibit cell cycle progression of cancer cells is determined by their ability to affect various molecular targets involved in regulating these processes (Fig. 9.4). Furthermore, these ITCs are also able to modulate these processes through their interaction with unique molecular targets dictated by their structure.

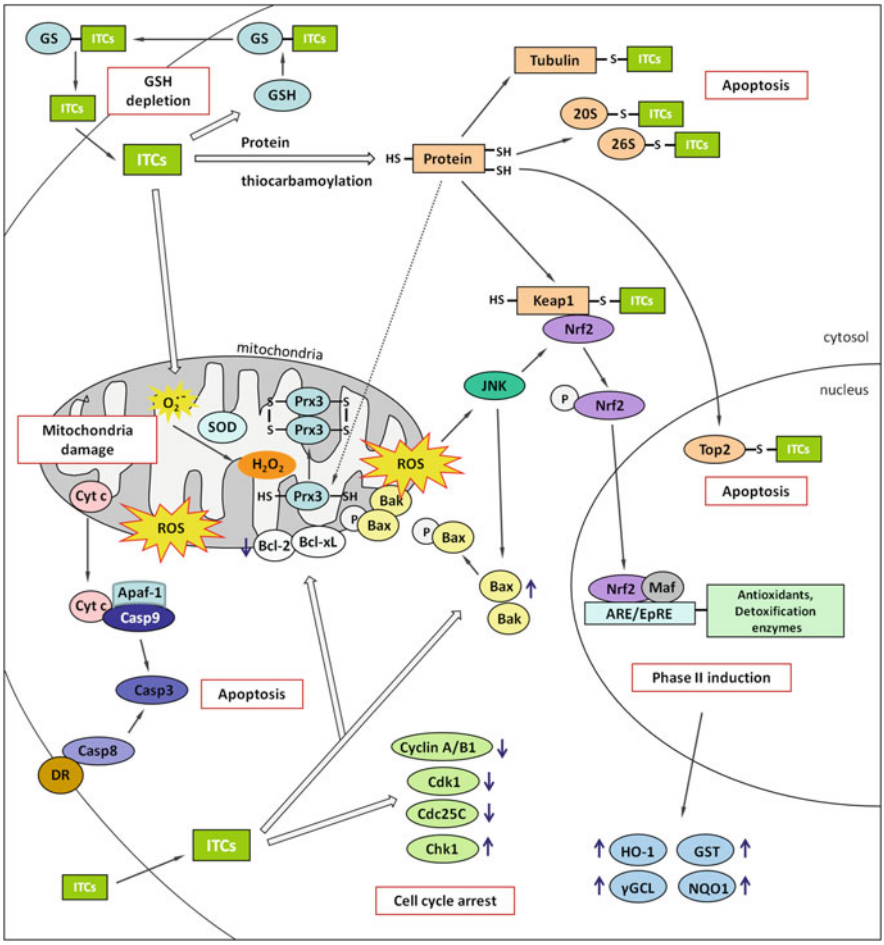


Fig. 9.4 Schematic representation of the signaling pathways contributing to ITC-induced cytotoxicity and indirect antioxidant effects (details are described in the text). *ITCs* isothiocyanates, *GSH* glutathione, *Top2* DNA topisomerase II, *Keap1* Kelch-like ECH-associated protein 1, *Nrf2* NF-E2 p45-related factor 2, *Maf* small musculoaponeurotic fibrosarcoma, *ARE/EpRE* antioxidant/electrophile response element, *γGCL* γ-glutamate-cysteine ligase, *GST* glutathione *S*-transferase, *HO-1* heme oxygenase 1, *NQO1* NAD(P)H:quinone oxidoreductase 1, *Bcl-2* B-cell lymphoma 2, *Bcl-xL* B-cell lymphoma-extra large, *Bak* Bcl-2 homologous antagonist killer, *Bax* Bcl-2–associated X protein, *SOD* superoxide dismutase, *Prx3* peroxiredoxin 3, *ROS* reactive oxygen species, *Cyt c* cytochrome c, *Apaf-1* apoptotic protease activating factor 1, *Casp3,8,9* caspase-3,-8,-9, *DR* death receptor, *JNK* c-Jun N-terminal kinase, *Cdk1* cyclin-dependent kinase 1, *Cdc25C* cell division cycle 25 homolog C, *Chk1* checkpoint kinase 1

9.4.1 Cellular Accumulation of ITCs and Depletion of Glutathione (GSH)

ITCs upon crossing the cell membrane accumulate in the cytosol as dithiocarbamates due to their rapid reaction with molecules containing thiol (-SH) group(s), or other nucleophilic moieties. As shown in Fig. 9.4, the main targets of ITCs for thiocarbamoylation are GSH and various cellular proteins. Intracellular conditions, such as high concentration of GSH (1–10 mM) and abundance of GSTs catalyzing the conjugation of GSH to the carbon of the –N=C=S moiety contribute to a rapid and highly efficient accumulation of ITCs. The intracellular concentration of ITCs can exceed the extracellular one over 100–200-fold within 1 to 3 h of exposure (Zhang 2000, 2001). Total intracellular BITC and PEITC accumulation levels were 1.04 and 0.66 mM, respectively, when UM-UC-3 cells were exposed to each ITC at 7.5 μM for 1 h (Tang and Zhang 2005). Then, as a means of detoxification, ITC-glutathione (ITC-SG) conjugates are transported out of the cell. Since the reaction between GSH and ITCs is reversible, the breakage of ITC-SG conjugates extracellularly promotes accumulation of ITCs and depletion of intracellular GSH exploited by the detoxification of ITCs re-entering cells. Measurements of GSH level in Jurkat T lymphoma cells upon 20 and 60 min of stimulation with 15 μM sulforaphane or PEITC showed a prominent reduction of GSH levels of around 60 and 30% of the control, respectively (Brown et al. 2008). Depletion of GSH, widely recognized as the main cellular redox buffer, can disrupt redox homeostasis and induce signaling pathways aimed at restoring the redox balance. Nevertheless, the cell survival/cell death fate is context dependent, and is contingent on drug concentration, duration of treatment, cell type (origin, normal vs. cancer cells) (Fig. 9.4) (Nakamura and Miyoshi 2010).

9.4.2 Apoptosis Induction by ITCs

One mechanism by which ITCs are able to wield their anti-cancer prowess is through the activation of apoptotic pathways. ITCs have been shown to suppress proliferation of different types of cancer cells (see Tables 9.5 and 9.6 for overview). Induction of apoptosis and cell cycle arrest by ITCs has been attributed to their pro-oxidative activity mediated via triggering mitochondrial signaling, as well as their electrophilic nature which causes the thiocarbamoylation of proteins of which the proper functioning is necessary for viability (Fig. 9.4). Multiple reports in the existing literature have shown that ITCs are potent inducers of apoptosis in a time- and dose-dependent manner. Interestingly, differences in molecular targets, mechanisms, and potency of apoptosis induction by ITCs have been identified. This underlines the importance of the side chain, unique to different compounds bearing an isothiocyanate moiety. Exemplary, the effectiveness of six different ITCs in dissipation of the mitochondrial membrane potential and apoptosis induction in leukemia cells (HL60) followed the

Table 9.5 Cytotoxicity attributed to pro-oxidative activities of ITCs

Organ	Cell line	ITCs concentration	Treatment time	Effect	References
Bladder	UM-UC-3	7.5, 15, 30 μM BITC, PEITC	3, 24 h	Procaspase-9 cleavage	Tang and Zhang (2005)
				PARP cleavage	
		7.5, 15, 30 μM BITC, PEITC, AITC, sulforaphane		Loss of MTMP	
				Cytochrome c release	
		7.5, 15, 30 μM BITC, PEITC		Damage of plasma membrane	
				Bak translocation to the mitochondria	
	Mitochondria from UM-UC-3	7.5, 15, 30 μM BITC, PEITC	30 min	Disturb association of Bcl-xL with both Bak and Bax	Tang and Zhang (2005)
		15, 50, 100 μM BITC, PEITC, AITC, sulforaphane		Loss of MTMP	
Bone	U2OS	5–15 μM BITC, PEITC	6–48 h	↓ Viable cells	Wu et al. (2011)
		5 μM BITC, 5 and 7.5 μM PEITC	48 h	G_2/M-phase cell cycle arrest	
		7.5 μM BITC, 10 μM PEITC	12, 18, 24 h	Apoptosis induction (sub-G_1 fraction)	
			24, 48 h	Chromatin condensation	
				DNA damage	
			0.5, 1, 2 h	↑ ROS	
			2, 4, 6 h	↑ NO	
				↑ Catalase level	
		7.5 μM BITC	6 h	↓ MnSOD level	
		7.5 μM BITC 10 μM PEITC	12, 24, 48 h	Loss of MTMP	
			12, 18, 24 h	↑ Cytochrome c, caspase-9, −3, AIF, cleaved PARP proteins	
			24 h	Cytochrome c and AIF translocation	

Brain	GBM8401	0.5–20 μM AITC	24 h	↓ Viable cells $IC_{50} = 9.25 \pm 0.69$ μM	Chen et al. (2010)
		10 μM AITC	24 h	G_2/M-phase cell cycle arrest	
				Apoptosis induction (sub-G_1 fraction)	
				Chromatin condensation	
			6, 12, 24 h	↓ Cdk1 activity	
			6, 12, 24, 48 h	↓ CDK1, cyclin B, cyclin A protein levels	
				↑ Cytochrome c, Apaf-1, pro-caspase-9, AIF, Endo G proteins in cytosolic fraction	
			24 h	↑ Caspase-3, −9 activity	
reast	MDA-MB-231, MCF-7	2.5–20 μM BITC, PEITC, sulforaphane	24 h	↓ Viable cells	Xiao et al. (2006)
		0.5, 1, 2.5 μM BITC	3, 6, 12, 24 h	G_2/M-phase cell cycle arrest	
				Apoptosis induction (sub-G_1 fraction)	
		2.5 or 10 μM BITC	1, 3, 6, 12 h	↓ Level of cyclin B1, Cdk1, Cdc25C	
		1, 2.5 μM BITC	24 h	Apoptosis induction (cells with condensed chromatin)	
		1, 2.5, 5, 10 μM BITC	24 h	Apoptosis induction (DNA fragmentation)	
		2.5 or 10 μM BITC	2–24 h	↑ Bax, Bak ↓ Bcl-2, Bcl-xL	
		2.5 or 5, 10 μM BITC	1, 2, 4, 6 h	Loss of MTMP	
		2.5 μM BITC	1, 3, 6, 12, 24 h	Cytochrome c release	
			6 h	Autophagososmes	
			1, 2, 4 h	↑ ROS	
			1, 3, 6, 12, 24 h	Procaspase-3, -9, -8 cleavage	

(continued)

Table 9.5 (continued)

Organ	Cell line	ITCs concentration	Treatment time	Effect	References
Breast	MDA-MB-231, MCF-7	2.5 μM BITC	2 h	↑ ROS	Xiao et al. (2008)
			24 h	Apoptosis induction (DNA fragmentation),	
				Procaspase-3 cleavage Caspase-3 activation	
			16, 24 h	Cytochrome c release	
			24 h	↓ Viable cells	
			6 h	Loss of MTMP	
			8, 16 h	Bax activation	
				PARP cleavage	
		5 μM BITC	1, 3, 6 h	↓ Complex III activity	
		2.5, 5 μM BITC	2, 4, 8, 16 h	↑ JNK, p38 MAPK activity	
	MCF7	5 μM BITC	24 h	↑Apoptosis induction	Sehrawat and Singh (2016)
		1–5 μM BITC	12	↓ Viable cells	
		5 μM BITC	6 h	↑ ROS	
	MDA-MB-361	5 μM BITC	24 h	↑ Apoptosis induction	
		5 μM BITC	6 h	↑ ROS	
Leukemia	Jurkat T lymphoma cells	2.5–60 μM PEITC	5–120 min	↑ Oxidation of mitochondrial Prx3	Brown et al. (2008)
		10–60 μM PEITC, Sulforaphane	1 h	↓ TrxR activity	
		10–60 μM PEITC, Sulforaphane	1 h	↓ GR activity	
		15 μM PEITC, Sulforaphane	20, 60 min	↓ GSH level	
		15 μM PEITC, BITC, AITC, Sulforaphane	24 h	↓ Viable cells	

	HL60, HL60/ADR, HL60/VCR[a]	0.5–25 μM AITC, BITC, PEITC, Sulforaphane, ERN, IBN	72 h	↓ Viable cells	Jakubikova et al. (2005b)
		5–20 μM AITC, BITC, PEITC, Sulforaphane, ERN, IBN	6, 24 h	G_2/M-phase cell cycle arrest	
				Apoptosis induction (sub-G_1 fraction)	
				Apoptosis and necrosis induction	
				Loss of MTMP	
Liver	RL34[b]	20, 50 μM BITC	8, 24 h	↓ Viable cells	Nakamura et al. (2002)
		20 μM BITC	8 h	Nuclear fragmentation	
				↑ Caspase-3 activity	
		5, 50 μM BITC	0.5 h	Loss of MTMP	
		5–100 μM BITC		↑ ROS	
Larynx	Hep-2	2.5–10 μM PEITC	24, 48, 72 h	↓ Viable cells	Dai et al. (2016)
		5, 7.5, 10 μM PEITC	24 h	Apoptosis induction	
		5, 7.5, 10 μM PEITC	48 h	↓ Cell invasion	
		7.5, 10 μM PEITC	24 h	G_2/M-phase cell cycle arrest	
Lung	L9981	1–40 μM BITC or PEITC	48 h	↓ Viable cells $IC_{50} = 5.0 \pm 0.22$ μM $IC_{50} = 9.7 \pm 0.39$ μM	Wu et al. (2010)
		5 μM BITC, 10 μM PEITC	30 h	↓ Cell migration (reduced to 8.1 and 16.5% of control)	
			24 h	↓ Cell invasion (reduced to 2.7 and 7.3% of control)	
			4 h	↓ mRNA of pro-metastasis genes: MMP-2, twist ↑ mRNA of anti-metastasis genes: β-catenin	
		5–20 μM BITC or PEITC	24 h	↓ MMP-2 and twist protein level	
		5 μM BITC, 10 μM PEITC	4 h	↑ ROS	
		5 or 10 μM BITC or PEITC	3 and 6 h	↓ GSH	
		5–20 μM BITC or PEITC	24 h	↓ Akt phosphorylation (activity)	
			18 h	↓ NF-κB transcriptional activation	
	L9981	7.5 or 10 μM BITC 12.5 or 20 μM PEITC	24 h	Apoptosis induction	Yan et al. (2011)

(continued)

Table 9.5 (continued)

Organ	Cell line	ITCs concentration	Treatment time	Effect	References
		7.5 μM BITC, 12.5 μM PEITC	24 h	G_2/M-phase cell cycle arrest	
		7.5 or 10 μM BITC 12.5 or 20 μM PEITC	24 h	↑ Phosphorylated JNK, p44/42 MAPK, p38	
Marrow	DA1-3b[c]	5, 10 μM PEITC	24 h	↓ Viable cells	Trachootham et al. (2006)
Myeloma	MM.1S, OPM1	1.6–50 μM PEITC, Sulforaphane	24, 48 h	↓ Viable cells	Jakubikova et al. (2011)
		2.5, 5, 10, 20 μM PEITC, Sulforaphane	12, 24, 48 h	G_2/M-phase cell cycle arrest	
				Apoptosis induction (sub-G_1 fraction)	
			12 h	Changes in level of cell cycle-related proteins	
			24, 48 h	↓ Viable cells (among death cells: ~30% necrotic, ~70% apoptotic)	
			24 h	Procaspase-3 cleavage, PARP cleavage	
			2, 6, 12, 24 h	Transient ↑ of JNK, c-Jun, MEK1, p38, ERK1/2, Akt, GSK3α/β	
	Bone marrow $CD138^+$ tumor cells	1.6–50 μM PEITC, Sulforaphane	48 h	↓ Viable cells	Jakubikova et al. (2011)
Ovary	T72Ras[d]	5, 10 μM PEITC	1, 3, 5 h	↑ ROS, ↑ NO	Trachootham et al. (2006)
		1–20 μM PEITC	1–48 h	↓ Viable cells	
		0.3–10 μM PEITC	10 days	↓ Cell proliferation, $IC_{50} = 0.49 \pm 0.1$ μM	
		10 μM PEITC	2, 4, 6, 8 h	↑ Cardiolipin oxidation	
				Loss of MTMP	
		5 μM PEITC	1, 3 h	↓ GSH	
		5, 10 μM PEITC	5 h	↓ GPX activity	
		5 μM PEITC	0.5, 1 h	↑ JNK activity	
	SKOV3	10 μM PEITC	6 h	↑ ROS	Trachootham et al. (2006)
		0.3–10 μM PEITC	10 days	↓ Cell proliferation	
		10 μM PEITC	23 h	↓ Viable cells	
Ovary	A2008	10 μM PEITC	24 h	↓ Viable cells	Trachootham et al. (2006)
		0.3–30 μM PEITC	3 days	↓ Cell proliferation	

	HEY	0.3–30 μM PEITC	3 days	↓ Cell proliferation	Trachootham et al. (2006)
	PA-1 SKOV3	1–40 μM PEITC	24, 48 h	↓ Viable cells PA-1 − IC_{50} = 7 (24 h), 5.09 (48 h) μM SKOV3 − IC_{50} = 7.95 (24 h), 4.67 (48 h) μM	Hong et al. (2015)
		5 μM PEITC	12, 24 h	↑ ROS	
		5 μM PEITC	12, 24 h	↑ UPR	
		5 μM PEITC	24, 48 h	↑ Apoptosis	
Skin	A375	5 μM PEITC, BITC, sulforaphane	48 h	G_2/M-phase cell cycle arrest	Mantso et al. (2019)
Prostate	LNCaP, PC-3	5 μM PEITC	2, 4, 6 h	↑ ROS	Xiao et al. (2010)
		2.5 μM PEITC	4 h		
		5 μM PEITC	6 h		
		2.5, 5 μM PEITC	24 h	Inhibition of complex III activity	
		5 μM PEITC	6 h	↓ Oxidative phophorylation rate	
				↓ Glycolysis rate	
			45 min	↓ ATP	
			4 h	Loss of MTMP	
		2.5 μM PEITC	8 h	Bax activation	
		5 μM PEITC	24 h	Apoptotic DNA fragmentation	
				Caspase-3 activation	
			6, 9 h	Autophagy induction	

ITCs isothiocyanates, *AITC* allyl ITC, *BITC* benzyl ITC, *PEITC* phenylethyl ITC, *ERN* erucin, *IBN* iberin, *PARP* poly (ADP-ribose) polymerase, *MTMP* mitochondrial transmembrane potential

[a]Multidrug resistant cells derived from HL60

[b]Rat liver epithelial RL34 cells

[c]Bcr-Abl transformed murine myeloid progenitor cells

[d]Ras-transformed ovarian epithelial cells

Table 9.6 Cytotoxicity attributed to electrophilic activities of ITCs

Organ	Cell line	ITCs conc.	Treatment time	Effect	References
Breast	MCF10A-Ras	BITC, PEITC, Sulforaphane	4 days	↓ Viable cells $IC_{50} = 3.2 \pm 0.7$; 3.4 ± 0.5; 10 ± 1.3	Lin et al. (2011)
		10 μM PEITC	6 h	↑ DNA damage	
		0.1 mM BITC, 1 mM BITC	1 and 5 min, 30 min	In vitro covalent binding to DNA topoisomerase II	
Lung	A549	1–100 μM BITC, PEITC, Sulforaphane	24 h	↓ Viable cells $IC_{50} = 13.8$; 18.3; 43	Mi et al. (2008)
		10 μM BITC or PEITC, 30 μM SFN	4–24 h	G_2/M-phase cell cycle arrest	
			24 h	Accumulation of mitotic cells	
		10 or 20 μM BITC, PEITC, Sulforaphane	24, 48, 72 h	Apoptosis induction (sub-G_1 fraction)	
			4–24 h	Caspase-3 activity	
		20 μM ^{14}C-PEITC, ^{14}C-SFN;	1 h	α- and β-tubulin binding	
		30 μM BITC, PEITC, Sulforaphane (in vitro)	Not stated	Tubulin polymerization inhibition	
		5 μM BITC or PEITC	0.5 or 1 h	Microtubule network disruption	
		20 μM	1 h	Covalent binding to cysteine residues in tubulin	
Myeloma Ovary Lung Prostate Colon Breast	U266 RPMI-8226 HeLa A549 PC-3 HT-29 MCF-7	10, 20, 30 μM BITC or PEITC	2–24 h	Inhibition of proteosome (26S and 20S) activity by direct binding	Mi et al. (2011)
Myeloma	U266	10 or 20 μM BITC, PEITC, SFN	1 h	↓ GSH	Mi et al. (2011)
		10 μM BITC or PEITC	24 h	G_2/M-phase cell cycle arrest	
		10 or 20 μM BITC, PEITC, SFN	24 h	Apoptosis induction (sub-G_1 fraction)	

(continued)

Table 9.6 (continued)

Organ	Cell line	ITCs conc.	Treatment time	Effect	References
		5, 10, 15 μM BITC	24 h	Apoptosis induction (PARP cleavage)	
		2.5–40 μM BITC	24, 48 h	↓ viable cells	
		10–40 μM BITC, PEITC, SFN	4 h	α- and β-tubulin aggregation and depletion	
Ovary	HeLa	10 μM BITC	20 h	Apoptosis induction (sub-G_1 fraction)	Mi et al. (2009)
		10 μM BITC	20 h	G_2/M-phase cell cycle arrest	

order: BITC = PEITC > ERN (erucin) = IBN (iberin) > AITC > sulforaphane (Jakubikova et al. 2005b). A 24-h exposure of human breast cancer cells to as little as 2.5 μM PEITC and BITC resulted in about 70% and 30% decrease in cell viability, respectively for MDA-MB-231 and MCF-7 cell lines (Xiao et al. 2006). The increase in each of the compound's concentration to 20 μM caused severe elevation in the number of dead cells (above 90%) in the case of PEITC and complete cell death with BITC. Sulforaphane, also used in this study, showed a relatively lower potential in inducing cell death. On the basis of analysis of DNA fragmentation, chromatin condensation, and percentage of cells in the sub-G_1 fraction, apoptosis induction was identified as a mechanism responsible for reduction of the number of viable cells (Xiao et al. 2006). Treatment of the same cell lines with low concentrations of BITC over 2–24 h resulted in an increase in the levels of the pro-apoptotic proteins Bax and Bak and down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL (Xiao et al. 2006). Interestingly, RNA interference of Bax and Bak conferred significant protection against PEITC-induced apoptosis (Xiao et al. 2010). Bcl-2 family members are known to influence the integrity of the mitochondrial membrane: the anti-apoptotic proteins are normally located at the mitochondrial membrane to protect its stability; the pro-apoptotic proteins translocate from the cytosol to the mitochondrial membrane in order to destabilize it in response to apoptotic stimuli. The results from a study performed in human bladder cancer cells (UM-UC3) showed ITC-mediated phosphorylation of Bcl-2, mitochondrial translocation of Bak, and disruption of the association of Bcl-xL with both Bak and Bax in the mitochondrial membrane, indicating that ITC-induced mitochondrial damage results at least in part from modulation of selected Bcl-2 family members (Tang and Zhang 2005). This effect was further complemented in breast cancer cells through the presence of the loss of mitochondrial membrane potential, cytochrome c release and finally activation of caspase-9, -3 and -8. Pre-treatment of cells with specific inhibitors for caspase-9 or -8 was associated with decreased cleavage of pro-caspase-3 and decreased DNA fragmentation, pointing at the involvement of both, the mitochondrial pathway (mediated by caspase-9) and the death receptor pathway

(mediated by caspase-8) in apoptosis induction (Xiao et al. 2006). The capacity of ITCs to induce apoptosis is also observed in various cell culture (Pappa et al. 2006, 2007a, b) as well as in vivo systems where researchers have linked this ability to the antineoplastic effects seen in their studies (Yeh et al. 2016a, b; Herz et al. 2014; Wang et al. 2014; Stan et al. 2014; Ni et al. 2013). For example, both BITC (Huang et al. 2018) and PEITC (Chou et al. 2018) have been found to inhibit the growth of xenograft tumors of glioblastoma multiforme in mice, in part, due to their capability to induce apoptosis in these cells through the down regulation of several anti-apoptotic proteins. In a highly metastatic human non-small cell lung cancer cell line L9981, BITC and PEITC have shown to potently induce apoptosis as well as cause the upregulation of the MAPK signaling pathway (a signaling pathway implicated in apoptosis induction), and that their effects were abrogated by the pretreatment with the anti-oxidant N-acetyl-cysteine (NAC) (Yan et al. 2011). Also, using the Affymetrix GeneChip microarray, treatment of these cells with 10 μM BITC for 24 h exhibited an upregulation of 77 and 52 genes involved in apoptosis and cell cycle progression respectively (Yan et al. 2011).

Another molecular mechanism by which these ITCs induce apoptosis could be through the modulation of the short form Recepteur d'Origine Nantais (sfRON), a receptor tyrosine kinase. Sehrawat and Singh reported that MCF7 cells overexpressing sfRON (MCF7/sfRON) treated with 5 μM BITC for 24 h had an approximately two-fold higher apoptotic-induction compared to the wild-type cells (Sehrawat and Singh 2016). MCF7/sfRON and MDA-MB-361/sfRON cells treated with 5 μM BITC for 6 h exhibited an increase in activated apoptotic proteins Bak and Bax compared to their respective wild-type counterparts (Sehrawat and Singh 2016). Also, it is interesting to note that MCF7/sfRON cells had increased basal ROS production and BITC-induced ROS production in these cells was significantly attenuated compared to their wild-type counterparts (Sehrawat and Singh 2016).

The combinatorial effects of ITCs in inducing cancer cell death has also been studied. It has been shown that in the non-small cell lung cancer cell line A549, the synergistic effects of AITC and sulforaphane led to a more extensive apoptosis induction compared to when the ITCs were singly administered (Rakariyatham et al. 2019). It will be interesting to evaluate the synergistic potential of ITCs for their antineoplastic potential in animal models.

Although there is a large body of evidence linking ITCs to apoptosis induction, it is noteworthy that there has been an instance where they have been implicated in the inhibition of apoptosis (Ho et al. 2012). In particular, in the left ventricle of the heart of a murine acquired immune deficiency syndrome (AIDS) model, Ho and colleagues found that sulforaphane, PEITC and BITC inhibit apoptosis by increasing the Bcl-2/Bax ratio when compared to the vehicle treated mice (Ho et al. 2012) and that mice treated with the ITCs survived at least 25 days longer than the control group.

9.4.3 The Role of ROS Induction in ITC-Mediated Cytotoxicity

Pro-oxidative properties of BITC, as potentially important for triggering apoptosis, were investigated by use of dihydroethidium (DHE) bromide (hydroethidine) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), fluorescent probes designed to indicate superoxide anion radicals and more general ROS levels, respectively (Table 9.5). MDA-MB-231 cells treated with 2.5 μM of BITC for 2 h were loaded with the dyes. Subsequent measurements of oxidized fluorescent products ethidium bromide and 2′,7′-dichlorofluorescein (DCF) fluorescence indicated increased signals for BITC-treated samples compared to a vehicle control. Moreover, apoptosis induction by BITC was significantly attenuated in the presence of combined superoxide dismutase and catalase mimetic EUK134, supporting the notion that ROS generation is critical for triggering the death pathway (Xiao et al. 2006). Further studies by the same authors demonstrated that increased ROS levels by BITC treatment were due to inhibition of Complex III of the mitochondrial respiratory chain (Xiao et al. 2008). BITC-induced ROS production and apoptosis were significantly inhibited by overexpression of the anti-oxidant enzymes catalase and Cu,Zn-superoxide dismutase and by pharmacological inhibition of the mitochondrial respiratory chain (Xiao et al. 2008). In accordance, the mitochondrial DNA-deficient Rho-0 variant of MDA-MB-231 cells was almost completely resistant to BITC-stimulated ROS generation and apoptosis induction (Xiao et al. 2008). Treatment with BITC caused activation of c-Jun N-terminal kinase (JNK) and the mitogen-activated protein kinase p38 (Xiao et al. 2008); the latter is s also observed upon treatment with PEITC (Dayalan Naidu et al. 2016). Pharmacological inhibition of both JNK and p38 ensured partial protection against BITC-induced apoptosis (Xiao et al. 2008). Concerning the cascade of events triggered by BITC, it can be concluded from this study that ROS production is up-stream of JNK and p38 activation, and such activation is up-stream of Bax conformational changes.

Hence, overexpression of catalase abolished activation of JNK and p38 in BITC treated cells, and BITC-mediated activation of Bax was suppressed by ectopic expression of a catalytically inactive mutant of JNKK2, which is a JNK specific kinase (Xiao et al. 2008). The importance of JNK, p38 and related signaling in mediating cytotoxic effects of sulforaphane and PEITC was examined in the MM.1S myeloma cell line (Jakubikova et al. 2011). Multiplex analysis of phosphorylation of diverse components of signaling cascades revealed transient changes in JNK, c-Jun, MEK1, p38, extracellular signal-regulated kinase (ERK)1/2, Akt, GSK3α/β and p53 activation in sulforaphane- and PEITC-treated cells, which may result from ITC-induced oxidative stress or potential targeting of phosphatases (Jakubikova et al. 2011). Studies done in human prostate cancer cells (LNCaP and PC-3) showed similarly that PEITC-induced cell death initiated by production of ROS (measured here by electron paramagnetic resonance spectroscopy: EPR) correlated with inhibition of Complex III activity, suppression of oxidative phosphorylation, and ATP depletion (Xiao et al. 2010). Pre-treatment of cells with 4 mM N-acetyl cysteine

(NAC) for 2 h, followed by further PEITC/NAC co-treatment, caused a decrease of ROS production and readouts for apoptotic markers (Xiao et al. 2010).

Nevertheless, a study by Mi et al. (2010) with use of [^{14}C]PEITC and [^{14}C] sulforaphane demonstrated that NAC pretreatment significantly reduced ITC cellular uptake by conjugating with ITCs extracellularly in the cell culture medium, suggesting that reduced uptake of ITCs, rather than the antioxidant activity of NAC itself, is responsible for the diminished downstream apoptotic effect (Mi et al. 2010). Therefore, other approaches, such as Rho-0 cells characterized by non-functional mitochondria or up-/down-regulation of antioxidant enzymes level would be more reliable to demonstrate the importance of ROS signaling induced by ITCs for downstream effects. Indeed, counterparts of LNCaP and PC-3 cells with overexpressed Mn-SOD or Cu,Zn-SOD, or unfunctional respiratory chain (Rho-0 cells), were more resistant to PEITC-mediated ROS generation and subsequent apoptosis induction (Xiao et al. 2010).

It is recognized now that the superoxide anion radical production by Complex III of the mitochondrial respiratory chain is directed towards both matrix and intermembrane space (Han et al. 2003). This would clarify why overexpression of SOD localized to the mitochondrial matrix (Mn-SOD) and SOD localized to the mitochondrial intermembrane space and cytosol (Cu,Zn-SOD) could decrease PEITC-mediated ROS generation (Xiao et al. 2010). On the other hand, there have been some studies reporting ITC-stimulated ROS-independent cell death (Wiczk et al. 2012; Hsu et al. 2013). Measuring [^{3}H]-leucine incorporation, Wiczk et al. observed that sulforaphane dose-dependently (10, 20 and 40 μM) reduced protein synthesis in PC-3 cells by 80, 50 and 20% when compared to the vehicle-treated cells (Wiczk et al. 2012). They further found that SFN-induced protein synthesis blockade occurred in a ROS-independent manner, and that cell death induced by SFN was due to the decrease in the levels of the short-lived protein survivin (Wiczk et al. 2012).

ROS accumulation activates the unfolded protein response (UPR), which causes ER stress, which if not alleviated, leads to activation of cell death pathways. In ovarian cancer cell lines SKOV3 and PA-1, exposure to 5 μM PEITC for 48 h caused cellular ROS accumulation, subsequent UPR activation and apoptotic cell death. These effects observed upon PEITC exposure were abrogated in the presence of the ROS scavenger NAC, signifying that PEITC-induced ROS is crucial for the UPR-induced apoptosis (Hong et al. 2015).

Studies by Brown et al. brought interesting insights into the exact topology of PEITC-mediated ROS generation (Brown et al. 2008). There exist several enzymes dedicated to remove intracellular hydrogen peroxide (H_2O_2), a product of superoxide dismutation by SOD. One type is represented by peroxiredoxins (Prxs) that in course of peroxide decomposition by specific cysteine residues generate a disulfide-linked intermolecular dimer. Hence, their oxidation can be analyzed by western blotting detection of a band shift from monomer (reduced Prx) to dimer (oxidized Prx). Interestingly, oxidation of mitochondrial peroxiredoxin 3 (Prx3) was detected as early as 5 min after exposure of Jurkat T lymphoma cells to PEITC. Time- (5–120 min with 15 μM) and dose- (2.5–60 μM for 60 min) dependent analyses

revealed that such oxidation is specific to the mitochondrial isoform, with cytoplasmic Prx1 and Prx2 remaining in their reduced forms (Brown et al. 2008). Treatment of the cells with sulforaphane under similar conditions had no influence on Prx3 dimerization. Moreover, the ability to disturb mitochondrial redox homeostasis by selected ITCs was correlated to their pro-apoptotic activity (from the most to least potent: PEITC, BITC, phenylhexyl ITC, phenylbutyl ITC, phenylpropyl ITC, AITC, sulforaphane) (Brown et al. 2008). In addition to regulation of Prx oxidation, ITCs are able to modulate cellular redox conditions by affecting the activity of other antioxidant enzymes, including thioredoxin reductase (Brown et al. 2008, 2010; Heiss and Gerhauser 2005), glutathione peroxidase (Trachootham et al. 2006), and glutathione reductase (Brown et al. 2008).

9.4.4 ITC-Related Electrophilicity and Apoptosis Induction

Cysteine residues present in diverse classes of proteins often have regulatory roles. They can be subjected to modifications such as oxidation, nitrosylation or glutathionylation, and also have the ability to bind to metals contained within proteins. Such cysteine modifications on proteins may have different biological consequences. Not all cysteine residues are equally reactive. Cysteine reactivity to electrophiles greatly varies across different proteins and also within the same protein. The protonation state of the cysteine residue determines the extent of its reactivity and nucleophilicity that is indicated by its pKa value, which in turn is affected by the proximate amino acids. Free cysteine residues usually have a pKa value of 8.6 whereas a reactive cysteine has a pKa value in the neutral or even acidic range, and thus typically exists in a thiolate form (Roos et al. 2013).

Keap1, the main negative regulator of transcription factor Nrf2 (Itoh et al. 1999), is equipped with highly reactive cysteines that serve as sensors for electrophiles (Dinkova-Kostova et al. 2002; McMahon et al. 2010; Saito et al. 2016), including ITCs, leading to Nrf2 stabilization and enhanced Nrf2-target gene expression (Fig. 9.4). Cysteine 151 in Keap1, which is surrounded by a cluster of basic amino acids (H129, K131, R135, K150, and H154), is the primary sensor for sulforaphane and PEITC (Zhang and Hannink 2003; Dayalan Naidu et al. 2018). However, at high concentration of PEITC (7.5 μM for immortalized mouse embryonic fibroblast cells), Nrf2 stabilization proceeds in the absence of cysteine 151 (Zhang and Hannink 2003; Dayalan Naidu et al. 2018), indicating that other cysteines are also modified within Keap1 as well as other proteins. Thus, mass spectrometric analyses have demonstrated that PEITC directly interacts with two cysteines of purified Prx3 in vitro (Brown et al. 2008). Also, by use of high-resolution mass spectrometry, it has been shown in vitro that PEITC modifies the single cysteine in GSTA1 as well as cysteines 14, 47 and 169 in GSTP1 causing the irreversible inhibition of the catalytic activity of these enzymes. This observation suggests that PEITC is capable of suppressing its own metabolism in the cells through its sulfhydryl reactivity (Kumari et al. 2016). It has been speculated that the inhibition of Complex III by PEITC may be caused by

covalent modification of critical sulfhydryl groups on subunit(s) of Complex III driven by the electrophilicity of the isothiocyanate moiety. In this regard, direct covalent modification of cellular proteins has been suggested to be an important early event in the induction of apoptosis by ITCs (Mi et al. 2007) (Table 9.6). So far, several specific protein targets have been identified (Mi et al. 2008, 2009, 2011; Lin et al. 2011). A study using two-dimensional gel electrophoresis of human lung cancer A549 cells treated with radiolabeled PEITC and sulforaphane revealed that tubulin may be an intracellular binding target for ITCs. The potency exerted by selected ITCs to cause mitotic arrest and apoptosis correlated positively with their ability to disrupt microtubule polymerization, with the established order of activity: BITC > PEITC > sulforaphane (IC_{50} = 13.8 > 18.3 > 43 μM) (Mi et al. 2008). Immunofluorescent microscopy showed disruption and degradation of the microtubule network in A549 cells treated with 5 μM of BITC or PEITC for 0.5 h and 1 h, respectively, while treatment with 10 μM sulforaphane over 4 h did not affect cells so potently. In contrast, exposure of cells to N-methyl phenethylamine, a structural analog of PEITC lacking the isothiocyanate functionality, did not interfere with tubulin polymerization, and consequently did not reduce cell viability (Mi et al. 2008). Additionally, tubulin precipitation was detected in BITC- and PEITC-treated cells, suggesting that this is a result of structural misfolding caused by ITCs. Further mass spectrometric data of tubulin purified from the insoluble fraction revealed that cysteine 347 of α-tubulin was covalently modified by BITC (Mi et al. 2008). The authors concluded that variation in ITCs activity to bind to tubulin and cause apoptosis results from the differences in their structure, which determines compound hydrophobicity, size, shape, and electrophilicity, all together influencing binding preferences (Mi et al. 2008). Exemplarily, the alkyl linkage joining the –N=C=S moiety to the aromatic ring is shorter in BITC then in PEITC. This might explain differences in covalent interaction between these ITCs and tubulin. Further studies by the same group demonstrated that ITCs can selectively induce degradation of both α- and β-tubulin in a variety of human cancer cell lines. Tubulin aggregation was found as the initial step in its proteasome-dependent degradation, which is triggered by ITC binding to tubulin and is independent from oxidative stress (Mi et al. 2009).

Other identified protein targets of ITCs are components of the 20S and 26S proteasomes. Their activities in cancer cells of different tissue origin were significantly inhibited by BITC or PEITC binding. This binding was unrelated to either ROS generation or ITC-induced protein degradation (Mi et al. 2011). Recent investigations have indicated that ITC-induced apoptosis of oncogene-transformed cells (MCF-10A-Ras) involved thiol modification of DNA topoisomerase II (Top2). siRNA-mediated knockdown of Top2α resulted in reduced sensitivity towards ITCs, showing that the Top2α protein level is important for mediating ITC-induced growth inhibition, DNA damage and apoptosis. In addition, proteomic analysis revealed that several cysteine residues on human Top2α were covalently modified by BITC, possibly contributing to formation of lethal Top2α-DNA covalent adducts (Top2α cleavage complex) (Lin et al. 2011).

MEK kinase 1 (MEKK1) is a MAP3K that regulates ERK and JNK MAPK pathways, pro-apoptotic and pro-survival pathways. Cross and colleagues found that

oxidative stress induced by menadione exposure inhibits the function of MEKK1 *via* the glutathionylation of its cysteine residue 1238 which is found in the ATP-binding domain (Cross and Templeton 2004). In a following study by the same group, MEKK1 overexpressed in CV-1 cells showed that 1 h exposure to PEITC from 50 μM to 200 μM showed reduction it is catalytic activity when the kinases were purified from these cells an assayed in an in vitro kinase assay. Importantly, using purified recombinant full length MEKK1 and the mutant MEKK1 C1238V which retains its wild-type kinase function whilst resistant to oxidative stimuli, it was shown that PEITC dose dependently (6.5 μM to 200 μM) caused a loss of kinase activity in the wild-type MEKK1 and that the mutant MEKK1 C1238V retained its kinase activity, indicating that C1238 is modified by PEITC thereby inhibiting its kinase activity (Cross et al. 2007). However, it remains to be seen whether inhibition of MEKK1 by PEITC affects its pro-apoptotic or its pro-survival activity. The MEK1/MEKK1/FLT3 inhibitor E6201 dose-dependently induced apoptosis in acute myeloid leukemia cells, therefore, it is possible that chemical inhibition of MEKK1 through PEITC may allow it to exert it pro-apoptotic effects.

Under basal conditions, the apoptosis signal-regulating kinase 1 (ASK1), a mitogen-activated protein kinase kinase kinase (MAP3K), is negatively regulated by the redox-sensitive protein thioredoxin 1 (Txn1) through direct interaction (Bishopric and Webster 2002). ASK1, is an upstream regulator of the MAPK family members p38 and JNK and when activated, signals pro-apoptotic pathways mediated by these kinases (Liu et al. 2000). Mutagenesis studies using purified recombinant proteins reveal that the Txn1 binds to ASK1 through its cysteines C32 and C35, where the former residue exhibits a relatively higher binding affinity (Kylarova et al. 2016). Oxidation of Txn1 has been implicated in its dissociation from ASK1 and the subsequent activation of the kinase (Nadeau et al. 2007; Saitoh et al. 1998). It has been found that in the hepatocellular carcinoma cell line MHCC97H, a 24 h exposure to 20 and 30 μM PEITC caused the reduction in the levels of reduced Txn1 and increased the levels of oxidized Txn1 in a dose-dependent manner (Zhang et al. 2012). In CV-1 cells expressing full length ASK1, exposure to PEITC does not affect its kinase activity (Cross et al. 2007). Since oxidation status of Txn1 is perturbed by PEITC, possibly through its electrophilic nature, it is highly conceivable that apoptosis induced by this isothiocyanate is mediated via ASK1 activation through the release of Txn1 from the kinase.

9.4.5 Necrosis, Autophagy, and Cell Cycle Arrest Triggered by ITCs

The cellular processes other than apoptosis triggered by ITCs-initiated oxidative stress or protein thiocarbamoylation deserve some attention and include induction of necrosis, autophagy, and cell cycle arrest. Such events triggered by ITCs can represent the responses interrelated to or distinct from apoptosis induction (Xiao et al. 2006).

Effects of sulforaphane and PEITC on induction of apoptosis and necrosis in myeloma cells (MM.1S) were quantified by flow cytometry upon staining with annexinV-FITC and PI (propidium iodide). The percentage ratio of apoptotic to necrotic death was about 70% to 30%, respectively (Jakubikova et al. 2011), demonstrating a significant contribution of necrosis to the overall mechanisms responsible for a drop of cell survival. The study performed in leukemia cells (HL60) treated with different ITCs for 6 h revealed an over 50% contribution of necrotic cells to the total pool of dead cells. This phenomenon was especially visible when higher concentrations of ITCs were used (Jakubikova et al. 2005b). Consistently, apoptosis was induced when rat liver cells were treated with 20 μM BITC, but increasing its concentration to 50 μM caused necrosis (Nakamura et al. 2002).

ROS resulting from the disturbance of the mitochondrial electron transport chain or catalase degradation can induce autophagy (Azad et al. 2009). Autophagy, a self-digestion process that degrades intracellular structures in response to stress caused by nutrient starvation, mitochondrial toxins, hypoxia, or ROS, can be involved in both cell survival and cell death. Autophagic degradation of cellular material generates amino acids and fatty acids, which can be used for protein synthesis and ATP generation during stressful conditions such as starvation. Autophagy also removes protein aggregates (which can trigger apoptosis) and damaged mitochondria (as a source of apoptotic proteins and toxic ROS). However, prolonged autophagy can lead to cell death through excessive self-digestion or activation of apoptosis (Azad et al. 2009; Kondo et al. 2005).

Xiao et al. investigated the induction of autophagy as an additional mechanism downstream of ROS generation by PEITC (Xiao et al. 2010). PEITC-initiated autophagy was partially dependent on ROS production in prostate LNCaP and PC-3 cancer cells, since their Rho-0 counterparts were less affected when autophagy markers were analyzed. Xiao et al. suggested that autophagy may represent a clearing mechanism for mitochondria involved in ROS production. Nevertheless, a possible influence of autophagy on apoptosis induction in this study could not be excluded and remains to be elucidated (Xiao et al. 2010). In triple negative breast cancer cell lines, MDA-MB-231, MDA-MB-468 and BT549, by the use of fluorescence microscopy techniques, it was reported that sulforaphane induced autophagy by downregulating HDAC6-mediated PTEN activation (Yang et al. 2018), where the latter is an important driver of autophagy (Ueno et al. 2008). BITC induced autophagy in the human colorectal cancer cells HCT-116 where increases in the lipidated form of LC3B and p62 which are proteins required for the formation of autophagic vesicles were observed (Liu et al. 2017). A similar effect was observed with the use of sulforaphane in the U2OS osteosarcoma cells (Olagnier et al. 2017) and PEITC in a prostate cancer mouse model (Powolny et al. 2011).

A common phenomenon caused by ITCs in different cancer cell lines is the inhibition of cell cycle progression. Most reports describe ITC-induced cell cycle arrest at the G_2-M phase. Statistically significant enrichment of the G_2-M fraction of MDA-MB-231 cells treated with 2.5 μM BITC was evident as early as 3 h after treatment and this effect was sustained, correlating with increased growth inhibition (Xiao et al. 2006). Studies by Jakubikova and colleagues showed that AITC (10 μM,

24 h) was the most potent inducer of G_2-M arrest among the six tested ITCs where 52% of the HL60 cells accumulated at the G_2-M phase (Jakubikova et al. 2005b). PEITC- and sulforaphane-induced G_2-M cell cycle arrest was accompanied by phosphorylation of histone H3 at serine 10 (a mitotic marker) in human myeloma cell lines (Jakubikova et al. 2011). Immunobloting analysis revealed that BITC-mediated cell cycle arrest was associated with a decrease in levels of proteins involved in regulation of G_2-M transition, including cyclin B1, cyclin-dependent kinase 1 (Cdk1), and cell division cycle 25C (Cdc25C) (Fig. 9.4) (Xiao et al. 2006). Expression patterns of cell cycle-related proteins were studied also in myeloma cells (MM.1S) and revealed a decrease of cyclin B1, p-Cdc2 and Cdc25C (Jakubikova et al. 2011). Similarly, the BITC- and PEITC-induced G_2-M phase arrest of human osteosarcoma U2OS cells was due to a reduction in cyclin A and B1 levels, accompanied by an increase of Chk1 and p53 levels, events that lead to G_2-M arrest (Wu et al. 2011). Along these lines, it has been demonstrated that treatment of glioma cells with AITC markedly reduced Cdk1/cyclin B activity and protein levels (Chen et al. 2010). Interestingly, experiments using phase-specific synchronized cells demonstrated that G_2-M phase-arrested cells are more sensitive to undergo apoptotic stimulation by BITC than cells in other phases (Miyoshi et al. 2004). A recent study conducted by Mantso and colleagues found that using low concentrations of sulforaphane, BITC and PEITC (5 μM) in the human melanoma cell line A375 for 48 h with a replenishment of cell growth media with the compounds after 24 h of exposure showed that these cells, in agreement with other independent reports in the literature, were arrested at the G_2-M phase (Mantso et al. 2019). BITC caused the highest proportion of A375 cells to arrest at G_2-M followed by sulforaphane and PEITC (Mantso et al. 2019). In this study, all three ITCs induced p21, p27, cyclin D1, cyclin D3, CDK2 and p53 phosphoserine 15 and caused a reduction in the cyclin dependent kinases (CDK) 4 and 6, where the levels of all of these proteins when perturbed cause cell cycle arrest (Mantso et al. 2019).

9.4.6 ITC-Mediated Selective Killing of Transformed and Cancer Cells

It is of great importance to address the question of whether normal cells are sensitive to ITC-induced oxidative and/or electrophilic stress and the following signals leading to cell death. Studies performed by Xiao et al. indicated that normal mammary epithelial cell lines (MCF-10A or HMEC) were significantly more resistant to growth arrest and apoptosis induction by BITC compared to breast cancer cell lines (MDA-MB-231 and MCF-7) (Xiao et al. 2006, 2008). Similarly, investigations done in human prostate cancer cell lines (LNCaP and PC-3) and their representative normal prostate epithelial cells (PrEC) revealed that ROS generation by PEITC is more harmful to cancer cells than to normal cells (Xiao et al. 2010). These results suggest that ITCs may selectively target cancer cells but spare normal breast or

prostate epithelium, which is a highly desirable property of potential anticancer agents (Xiao et al. 2006, 2010).

In ovarian cancer cell lines SKOV3 and PA-1, exposure to PEITC induced cell death, however, in the same study, this effect of PEITC was not observed in normal ovarian epithelial cells (Hong et al. 2015). Similarly, in human laryngeal carcinoma Hep-2 cells, exposure to a maximum of 10 μM PEITC exerted its anti-cancer properties by causing cell cycle arrest at G_2-M, inhibiting cell proliferation and inducing apoptosis, and these effects were not observed in the normal human bronchial epithelial cells 16HBE. This finding shows the sensitivity of the Hep-2 cells to PEITC compared to the normal cells (Dai et al. 2016). Furthermore, findings from this study provide a therapeutic strategy where a safe range (up to 10 μM in vitro) of administration of PEITC will confer minimal toxicity to normal cells and at the same time has the ability to exert its antineoplastic effects on cancer cells (Dai et al. 2016).

Trachootham et al. developed an interesting model, consisting of immortalized ovarian epithelial cells (T72) and their H-Ras^{V12} transformed counterparts (T72Ras), to test the concept that increased ROS generation associated with oncogenic transformation may serve as a biochemical basis to selectively kill cancer cells using agents that cause further oxidative stress (Trachootham et al. 2006). Indeed, oxidative stress facilitated by exposure to PEITC was significantly more pronounced in T72Ras cells comparing to parental T72 cells, corresponding with a lowered survival of transformed cells in response to treatment (10 days treatment: $IC_{50} = 0.49 \pm 0.1$ μM for T72Ras vs. $IC_{50} = 1.95 \pm 0.1$ μM for T72) (Trachootham et al. 2006). The selective killing of Ras-transformed cells by PEITC was attributed to ROS-mediated damage of mitochondria. PEITC was also proved to be effective in killing naturally occurring human ovarian cancer cells (SKOV3, A2008, HEY) and exhibited significant therapeutic activity in vivo by prolonging survival of mice bearing Ras-transformed ovarian cancer cells (Trachootham et al. 2006). Similar pro-survival effects combined with decreased tumor volume were achieved with sulforaphane and PEITC in a myeloma xenograft mouse model (Jakubikova et al. 2011). As important factor for therapeutic applications, and shown in later studies, ITCs concentrations required to produce statistically significant inhibition of cancer cell growth may be achievable in vivo.

9.5 Summary

Isothiocyanates are promising multitarget cancer preventive agents. They exert health promoting effects mainly through induction of cytoprotective enzymes or selective toxicity towards cancer cells, processes critical for decreasing the risk of cancer onset and retardation or inhibition of tumor growth, respectively. Various investigations performed in cultured cancer cells support the notion that pro-oxidative and electrophilic activities of ITCs serve as a main driving force of their anti-tumor properties. The electrophilic nature of ITCs determines their

targeting to molecules containing nucleophilic moieties. Indeed, upon entering cells ITCs are metabolized by conjugation with the cysteine residue of GSH, and due to extracellular exclusion of such conjugates during the detoxification process, cause depletion of this main cellular redox buffer. This, together with direct reactivity with cysteine residues within their target proteins and oxidative stress mediated *via* the mitochondrial pathway, challenges the cellular anti-oxidant defense. Such conditions stimulate the response of redox-sensitive proteins. One of them is Keap1, which upon sensor cysteine modification by ITCs loses its ability to target Nrf2 for ubiquitination and proteasomal degradation, enabling Nrf2 to accumulate, translocate to the nucleus and act as a transcription factor for cytoprotective genes regulated via ARE/EpRE. Subsequent increase of cytoprotective enzymes determines the restoration of the GSH pool and re-balancing of the cellular redox homeostasis. Nevertheless, this pro-survival signaling can be confronted by cell death-promoting pathways that are turned on in response to ITC-mediated cellular stress. Thiocarbamoylation of proteins, such as tubulin, proteasome or topoisomerase II, has been demonstrated as an early and critical event for induction of apoptosis and cell cycle arrest by ITCs. Similar reactivity of ITCs toward Complex III of the mitochondrial respiratory chain has been suggested to trigger ROS production and further mitochondrial damage, contributing to caspase-executed apoptosis.

Undoubtedly, ITCs possess potential to exhibit various biological activities. This renders this family of compounds highly effective in providing protection against cancer in animal models, induced by a variety of chemical carcinogens. In addition to preventing chemically induced cancers, several ITC compounds have also been shown to inhibit growth of cancer cells in vivo. In translating the anti-cancer efficacy of ITCs into the clinic, combinatorial therapy has been suggested whereby chemopreventive compounds are given in association with drugs currently used in chemotherapy, to achieve synergistic interaction for anti-cancer activity and reduce harmful effects (Russo et al. 2010).

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