Vegetable-derived isothiocyanates: anti-proliferative activity and mechanism of action

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Many isothiocyanates (ITC), which are available to human subjects mainly through consumption of cruciferous vegetables, demonstrate strong cancer-preventive activity in animal models. Human studies also show an inverse association between consumption of ITC and risk of cancer in several organs. Whereas earlier studies primarily focused on the ability of ITC to inhibit carcinogen-activating enzymes and induce carcinogen-detoxifying enzymes, more recent investigations have shown that ITC inhibit the proliferation of tumour cells both in vitro and in vivo by inducing apoptosis and arresting cell cycle progression. ITC cause acute cellular stress, which may be the initiating event for these effects. These findings shed new light on the mechanism of action of ITC and indicate that ITC may be useful both as cancer-preventive and therapeutic agents. ITC activate caspase 9-mediated apoptosis, apparently resulting from mitochondrial damage, and also activate caspase 8, but the mechanism remains to be defined. Cell cycle arrest caused by ITC occurs mainly in the G2/M phase, and both the G2 and M phases are targetted; critical G2-phase regulators, including cyclin B1, cell division cycle (Cdc) 2 and Cdc25C, are down regulated or inhibited, and tubulin polymerization and spindle assembly are disrupted. Moreover, ITC are metabolized in vivo through the mercapturic acid pathway, giving rise to thiol conjugates (dithiocarbamates). Studies show that these dithiocarbamates are similar to their parent ITC in exerting anti-proliferative activity. Taken together, dietary ITC are highly-promising anti-cancer agents, capable of targeting multiple cellular components that are important for tumour cell survival and proliferation.

Isothiocyanate: Anti-cancer agent: Cancer chemopreventive agent

All isothiocyanates (ITC) are characterized by the presence of a –N=\(\text{C}=\text{S}\) group, and many lines of evidence indicate that the biological activities of ITC may be primarily mediated through the reaction of the electrophilic central C of –N=C=S with cellular nucleophilic targets. Since \(\alpha\)-napthyl ITC was first shown to inhibit liver tumourigenesis induced by chemical carcinogens in rats about 40 years ago (Sasaki, 1963; Sidransky et al. 1966), numerous studies have not only confirmed these early findings but have also uncovered the cancer-preventive activities of other ITC (Zhang & Talalay, 1994; Hecht, 2000). Of particular importance is the finding that many of the ITC that show strong tumour-inhibitory effects in rodents are present in commonly-consumed cruciferous vegetables, such as broccoli, cabbage and watercress (Nasturtium officinale), and are therefore widely consumed by human subjects. Indeed, several recent epidemiological studies have reported an inverse association between consumption of dietary ITC and cancer risk in several organs (London et al. 2000; Spitz et al. 2000; Zhao et al. 2001; Seow et al. 2002; Fowke et al. 2003). These findings have provided an explanation for the widely-recognized cancer-preventive activity of cruciferous vegetables, i.e. ITC may be part of the active ingredients. Interestingly, available evidence suggests that ITC are probably absent in normal or fresh plants (Fahey et al. 1997) but are rapidly generated from \(\beta\)-thioglucoside \(\text{N}\)-hydroxysulfates (glucosinolates) through a hydrolysis reaction catalysed by the coexisting myrosinase (thioglucoside glucohydrolase) on damage of plant cells (Fenwick et al. 1983). Many cruciferous vegetables are abundant in certain glucosinolates, but the compounds are segregated from myrosinase in normal plant cells.

Although early studies mainly focused on the ability of ITC to inhibit the formation of cancer cells, i.e. inhibition of carcinogenesis, it is now well-known that ITC can also
suppress the survival and proliferation of existing cancer cells (the anti-cancer activity). The molecular basis for both the anti-carcinogenic and anti-cancer effects of ITC has been extensively investigated, showing that ITC can target cancer in multiple directions, including inhibition of carcinogen-activating enzymes, induction of carcinogen-detecting enzymes, induction of apoptosis and arrest of cell cycle progression, as well as other mechanisms that are not yet well understood (Conaway et al. 2002; Kaum et al. 2004; Zhang, 2004; Zhang et al. 2005). Among these mechanisms, however, induction of apoptosis and cell cycle arrest are of particular interest as they may be exploited for both cancer prevention and cancer treatment. The present review will examine current knowledge about the impact of dietary ITC on the survival and proliferation of cancer cells and their modulation of apoptosis and cell cycle progression.

**Anti-proliferative effects of dietary isothiocyanates**

Numerous studies have reported the inhibitory effects of ITC on the growth of both animal and human cancer cells in culture. Overall, these studies show that ITC inhibit cell growth at low micromolar concentrations and their activities are not cell specific. Moreover, ITC may selectively target transformed or malignant cells, as they are less potent against the growth of normal cells (Musk & Johnson, 1993; Gamet-Payrastre et al. 1998; Srivastava et al. 2003; Choi & Singh, 2005). There are other anti-proliferative properties of ITC that are also highly interesting. First, exposure of cells to ITC, including allyl ITC (AITC), benzy1 ITC (BITC) and phenethyl ITC (PEITC) for only 3 h is long enough to inhibit cell growth, indicating a rapid and irreversible interaction of these ITC with cellular targets (Zhang et al. 2003; Tang & Zhang, 2004). This finding is important because it suggests that the anti-proliferative activity of the compounds may not be markedly affected by their rapid disposal kinetics in vivo (Ioannou et al. 1984; Ye et al. 2002; Ji & Morris, 2003). Second, drug-resistant cancer cells that over-express multidrug-resistance-associated protein-1 and P-glycoprotein-1 are sensitive to ITC (Zhang et al. 2005). In this context it is noteworthy that Morris and co-workers (Hu & Morris, 2004; Ji & Morris, 2004) have recently reported that ITC are inhibitors of multidrug-resistance-associated protein-1, P-glycoprotein-1 and other drug transporters. Third, metabolic conversion of ITC in vivo may not markedly affect the anti-proliferative activity of these compounds. ITC are metabolized in vivo primarily through the mercapturic acid pathway, where an initial conjugation of ITC with glutathione (GSH) gives rise to the corresponding GSH conjugates, which then undergo further enzymic modifications to form sequentially the cysteinylglycine, cysteine and N-acetylcysteine (NAC) conjugates, which are excreted in the urine (Brusewitz et al. 1977). It has been shown (Xu & Thornalley, 2000; Zhang et al. 2003) that the conjugates display potencies very similar to their parent ITC in inhibiting the growth of cancer cells. These conjugates (generally termed dithiocarbamates) are considered to be the carriers of ITC, because they are unstable and readily dissociate to the parent ITC (Conaway et al. 2001).

Several ITC have also been shown to inhibit the growth of cancer cells in vivo. Singh and co-workers (Srivastava et al. 2003; Singh AV et al. 2004; Xiao et al. 2005) have reported that the growth of mouse or human prostate cancer cells that are subcutaneously inoculated in mice is retarded by AITC (10 μmol per mouse by intraperitoneal injection, three times per week), PEITC (9–12 μmol per mouse by oral intubation three times per week) or sulforaphane (SF; 5.6 μmol/kg diet daily). Moreover, Chiao et al. (2004) have also reported that oral administration of the NAC conjugate of PEITC (8 μmol/g diet daily) inhibits the proliferation of human prostate cancer cell xenografts in mice. Tumours harvested from mice treated with the ITC or the PEITC metabolite show a decrease in cell proliferation and an increase in apoptosis. These results clearly show the in vivo anti-cancer activity of dietary ITC and suggest that they are potentially useful for treating prostate cancers in man.

**Isothiocyanates cause acute cellular stress**

ITC are dichotomous modulators of cellular stress and cell survival; exposure of cells to ITC rapidly leads to an increase in stress and perhaps cell death but elicits a delayed induction of antioxidative and anti-carcinogenic enzymes and increased detoxification capacity (Zhang et al. 2005). While the induction of cellular protective enzymes and detoxification capacity by ITC will not be reviewed here, as it does not directly relate to the anti-proliferative activities of the compounds and much information is available elsewhere (Zhang, 2004; Kaum et al. 2004; Zhang et al. 2005), understanding how ITC cause cellular stress may be key to the elucidation of the mechanism responsible for their anti-proliferative effects.

**Isothiocyanates cause alkylation and depletion of cellular glutathione**

Most ITC are electrophiles because of the presence of a N=C=S group, which can react with various nucleophiles, especially those that are S-based (thiocarbamoylation). Indeed, the strong reaction of ITC with cellular thiols, particularly GSH (the most abundant intracellular thiol), is primarily responsible for the rapid accumulation of ITC in cells, which has recently been observed in the authors’ laboratory (Fig. 1). For example, when murine hepatoma Hepa1c1c7 cells are exposed to SF or BITC at 50 μM for 30 min the total intracellular ITC accumulations are 5.1 and 5.9 μM respectively, a 110–190-fold increase over the extracellular ITC concentration, but 98% SF and 62% BITC are present in the cell as GSH conjugates (Zhang, 2000). ITC appear to penetrate a cell by diffusion, but once in the cell they are rapidly conjugated with GSH and other thiols (Zhang, 2001). Not surprisingly, under the conditions described intracellular GSH concentration drops from 7.3 mM to 3.3–3.7 mM (49–55% depletion; Zhang, 2000). Similar effects of these and other ITC on GSH have been reported in other cells (Xu & Thornalley, 2001; Kim et al., 2002).
et al. 2003; Hudson et al. 2005). Moreover, because ITC conjugated with GSH are rapidly removed by membrane efflux pumps (Zhang & Callaway, 2002; Callaway et al. 2004), and the expelled conjugates may release ITC for re-accumulation in cells (Zhang & Callaway, 2002), the depletion of GSH by ITC may be especially marked and efficient (Fig. 1). Depletion of GSH undoubtedly renders cells more susceptible to stress and stress-induced injury and may trigger apoptosis (Hall, 1999). However, the rapid and dramatic alkylation and depletion of GSH by ITC is largely transient and contrasts with a delayed but prolonged increase in GSH levels in ITC-treated cells (Zhang & Talalay, 2000, 2002; Payen et al. 2001; Rose et al. 2003; Tang & Zhang, 2005; Xiao et al. 2005). Cell exposure to ITC apparently causes damage to both outer and inner mitochondrial membranes, as both cytochrome c (normally residing in the inter-membrane space) and malate dehydrogenase (normally residing in the mitochondrial matrix) are detected in the cytosolic fraction after ITC treatment (Nakamura et al. 2002; Tang & Zhang, 2005). Moreover, treatment of isolated mitochondria with GSH conjugates of ITC (the principal ITC metabolites in cells) leads to mitochondrial damage, indicating that these conjugates may be the primary cause of mitochondrial damage in ITC-treated cells (Tang & Zhang, 2005). The exact mechanism by which ITC or their GSH conjugates damage mitochondria remains largely unknown. Several synthetic ITC, including p-bromophenyl ITC, 4,4'-diisothiocyanatebiphenyl and β-naphthylmethyl ITC, have been shown to uncouple oxidative phosphorylation (Miko & Chance, 1975), but it is not known if any dietary ITC have the same effect. Bcl-2 family proteins are known to either stabilize (Bcl-2, Bcl-xl and Mcl-1) or destabilize...
(Bad, Bak, Bax and Bid) the mitochondrial membrane. Singh and co-workers (Choi & Singh, 2005; Xiao et al., 2005) have recently shown that cells deficient in Bax and/or Bax are resistant to death induced by SF and PEITC. The known effects of ITC on Bcl-2 family proteins include: inactivation of Bcl-2 via its phosphorylation by c-Jun N-terminal kinase; down-regulation of Bcl-2, Bcl-xl and Mcl-1; activation of Bid; up-regulation of Bad, Bak and Bax; increased mitochondrial translocation of Bax (Xu & Thornalley, 2001b; Xiao et al., 2003; Miyoshi et al., 2004; Srivastava & Singh, 2004; Singh SV et al., 2004; Xiao et al., 2004; Choi & Singh, 2005; Rose et al., 2005; Tang & Zhang, 2005; Xiao et al., 2005). Moreover, it has recently been found (Tang & Zhang, 2005) that treatment of mitochondria isolated from human bladder cancer UM-UC-3 cells with BITC disrupts the association of Bcl-xl with Bad and Bax. However, available information suggests that these effects are somewhat cell specific. For example, treatment of HepG2 cells with 20 μM PEITC causes the level of cytosolic Bax to increase, as well as translocation of Bax to the mitochondria (Rose et al., 2005), whereas treatment of murine prostate cancer cells (TRAMP-C1 and TRAMP-C2) with 10 μM PEITC either does not affect (TRAMP-C1) or actually reduces (TRAMP-C2) the Bax protein level and does not affect Bax mitochondrial translocation in either cell line (Xiao et al., 2005). Mitogen-activated protein kinases may also play a role in ITC-induced mitochondrial damage, as described later.

Since reactive oxygen species (ROS) are produced in the mitochondria and may leak out of damaged mitochondia, it is not surprising that many studies have shown an increase in intracellular ROS levels in ITC-treated cells. Indeed, ROS generation seems to correspond with mitochondrial damage in ITC-treated cells. For example, mitochondrial damage and ROS generation are detected after incubation of rat liver RL34 cells with 5–10 μM BITC for 30 min (Nakamura et al., 2000). Also, while an increase in both superoxide and H2O2 are detected in ITC-treated cells, H2O2 seems to be derived from dismutation of superoxide, which mainly occurs in mitochondria (Nakamura et al., 2002). Moreover, similar to mitochondrial damage caused by ITC, ROS generation by ITC is neither cell nor ITC specific (Payen et al., 2001; Rose et al., 2003; Xiao et al., 2005). While their excessive production probably causes non-specific cytotoxicity, ROS appear to play an important role in apoptosis (Buttke & Sandstrom, 1994) and cell cycle arrest (Shackelford et al., 2000). In fact, SF-induced cell death in human prostate cancer cells is initiated by increased ROS production (Singh et al., 2005), and PEITC-induced apoptosis in human leukaemia Jurkat T cells and HL60 cells is inhibited by antioxidants, including NAC and GSH (Chen et al., 1998; Xu & Thornalley, 2001a).

Isothiocyanates induce apoptosis

Induction of apoptosis by ITC was first reported by Tan and co-workers (Chen et al., 1998), who showed that BITC and PEITC at 5 μM induces apoptosis in Jurkat cells through a c-Jun N-terminal kinase-mediated mechanism. Since then, numerous studies have shown the apoptosis-inducing activities of a large number of ITC in a variety of cancer cell lines in culture and tumour xenografts in mice in vivo. ITC-treated cells frequently show activation of two initiator caspases, caspase 8 and caspase 9, indicative of activation of both death receptor signalling and mitochondria signalling (Fig. 1), although it is often difficult to know which signalling pathway may play a more important role in ITC-induced apoptosis. The majority of studies, however, have focused on the mitochondria-mediated apoptosis pathway. Mitochondria-mediated apoptosis is initiated when cytochrome c is released from the mitochondrial inter-membrane space into the cytoplasm, where together with apoptosis protease activation factor-1 it recruits and activates caspase 9.

Activated caspase 9 in turn activates effector caspases, including caspase 3, leading to apoptotic cell death. In line with the finding that treatment of cells with ITC causes mitochondrial damage, as described earlier, many studies (for example, see Nakamura et al., 2002; Hu et al., 2003; Zhang et al., 2003; Rose et al., 2005; Tang & Zhang, 2005; Xiao et al., 2005) have reported the release of cytochrome c from mitochondria to the cytoplasm as well as activation of caspase 9 and caspase 3 in ITC-treated cells. Moreover, studies in both human leukaemia HL60 cells and UM-UC-3 cells (Zhang et al., 2003; Tang & Zhang, 2005), as well as in murine embryonic fibroblasts (Choi & Singh, 2005), have shown that mitochondrial release of cytochrome c and activation of caspase 9 and caspase 3 occur as early as 1–3 h after cell exposure begins, coinciding with the time when mitochondria are damaged by ITC.

Current knowledge about the role of Bcl-2 family proteins in mediating ITC-induced apoptosis is incomplete. Nevertheless, a number of Bcl-2 family proteins that have been shown to be modulated by ITC (as described earlier) and may play a role in ITC-induced apoptosis, including Bcl-2, Bcl-xl, Bad, Bak and Bid, probably exert their effects through mitochondria (Chen et al., 1998; Xu & Thornalley, 2001b; Hu et al., 2003; Choi & Singh, 2005; Xiao et al., 2005). The three mitogen-activated protein kinases, including c-Jun N-terminal kinase, extracellular signal-regulated protein kinases and p38 kinases, have also been shown to be rapidly activated by ITC and contribute to apoptosis in a somewhat cell-specific fashion (Xiao & Singh, 2002; Hu et al., 2003; Lui et al., 2003; Miyoshi et al., 2004). Information on exactly how the mitogen-activated protein kinases mediate ITC-induced apoptosis is unavailable, but it seems likely that mitochondria are involved in their actions: c-Jun N-terminal kinase is known to inactivate Bcl-2 (Li et al., 2005); both extracellular signal-regulated protein kinases and p38 can phosphorylate p53, which in turn modulates Bax (Miyashita & Reed, 1995); extracellular signal-regulated protein kinases may also modulate Bad (Scheid et al., 1999). The role of p53 in ITC-induced apoptosis, however, is also cell specific, as it has been reported to be essential in PEITC-induced apoptosis in JB6 mouse epidermal cells (Huang et al., 1998), but PEITC potently induces apoptosis in both p53-deficient PC-3 human prostate cancer cells (Xiao & Singh, 2002) and p53-deficient HL60 cells (Xu & Thornalley, 2000).
ITC may also modulate other factors involved in the mitochondria-mediated apoptosis. Recently, it has been shown (Choi & Singh, 2005) that when murine embryonic fibroblasts are incubated with SF for 8–24 h: Smac/DIABLO, which is known to counter caspase inhibitors, is activated (released from mitochondria to cytoplasm); apoptosis protease activation factor-1, which facilitates caspase 9 activation by cytochrome c, is up regulated; X-linked inhibitor of apoptosis, which inhibits caspases, is down regulated. However, whether these changes also occur in other cells, or with other ITC, remains to be investigated.

Caspase 8 is another initiator caspase that is frequently activated by ITC (Xu & Thornalley, 2000; Zhang et al. 2003; Pham et al. 2004; Tang & Zhang, 2004, 2005; Xiao et al. 2004). Activated caspase 8 can directly activate effector caspases such as caspase 3 (Thornberry & Lazebnik, 1998), or cleave (activate) Bid, which then interacts with Bcl-xl and causes mitochondrial release of cytochrome c (Li et al. 1998). Indeed, Xu & Thornalley (2001b) have shown that treatment of HL60 cells with 10 μM PEITC for 6 h results in activation of Bid. However, how ITC activate caspase 8 is presently unknown, although caspase 8 is known to be downstream of death receptor signalling (Ashkenazi & Dixit, 1998). Interestingly, treatment of a number of cell lines with either AITC or SF has recently been shown to result in increased histone deacetylation, apparently a result of inhibition of histone deacetylase (Lea et al. 2001; Myzak et al. 2004). Non-ITC histone deacetylase inhibitors have been shown to induce the expression of death receptor signalling (both death receptor and their ligands are up regulated) in leukaemia cells by increasing promoter region histone acetylation (Insinga et al. 2005; Nebbioso et al. 2005). Whether ITC activate caspase 8 through inhibition of histone deacetylase remains to be investigated. However, Singh et al. (2005) have recently reported that SF treatment of PC-3 cells leads to an increase in Fas level (one of the death receptor family members), accompanied by activation of caspase 8 and Bid.

Isothiocyanates arrest cell cycle progression

The ability of an ITC to arrest cell cycle progression was first reported by Hasegawa et al. (1993), who showed that HeLa cells are arrested in the G2/M phase after incubation with AITC, BITC or PEITC at 2.5–10 μM for 16 h. Since then, these and other ITC, as well as particular metabolites of ITC (NAC conjugates), have been repeatedly shown to arrest cell cycle progression in a wide variety of cell lines. Whereas the majority of cell lines tested are arrested by ITC in the G2/M phase, in a few cell lines the same ITC cause G1-phase or S-phase arrest (Chiao et al. 2000; Zhang et al. 2003; Tang & Zhang, 2004). Little is known about the differential cell cycle arrest effects among cell lines. In this context, however, it is of interest to note that both human prostate cancer LNCaP cells (androgen-dependent and p53-intact) and DU-145 cells (androgen-independent and p53-mutated) are arrested by the NAC conjugate of PEITC in the G1 phase (Chiao et al. 2000), whereas PC-3 cells (androgen-independent and p53-mutated) are arrested by PEITC in the G2/M phase (Xiao et al. 2004). The differential effects are probably not a result of different behaviours of PEITC and NAC–PEITC, as NAC conjugates of ITC are known to exert their effects by releasing ITC. Indeed, both SF and its NAC conjugate block LNCaP cells in the G1 phase (Chiao et al. 2002), but SF blocks PC-3 cells in the G2/M phase (Singh SV et al. 2004).

G1-phase arrest of LNCaP and DU-145 cells treated with SF, NAC–SF or NAC–PEITC is associated with either increased p21 expression or decreased cyclin D1 expression (Chiao et al. 2000, 2002). p21 is a negative G1-phase regulator and cyclin D1 is a positive G1-phase regulator, suggesting that these proteins may mediate G1-phase arrest by SF and the NAC–ITC conjugates (Fig. 1). Treatment of UM-UC-3 cells with AITC, BITC or PEITC causes S-phase arrest, in addition to G2/M-phase arrest (Tang & Zhang, 2004), and the expression levels of cyclin A and cyclin-dependent kinase 2 (the primary S-phase regulators) appeared to be reduced (Fig. 1). Although in the majority of published papers the impact of ITC on the G1 and M phases has been described indiscriminately, it is now increasingly evident that ITC arrest cells in both the G2 and M phases by targeting corresponding regulators. The cyclin B–cell division cycle (Cdc) 2 complex plays a critical role in cell transition from the G2 phase to the M phase (Shackelford et al. 2000), but it requires activation by Cdc25C, a phosphatase that removes the inhibitory phosphates on T-14/Y-15 of Cdc2 (Shackelford et al. 2000). Published data have shown that ITC can target all three proteins (Fig. 1). For example, PEITC in HepG2 cells down regulates Cdc2 (Rose et al. 2003) and BITC and PEITC down regulate both cyclin B1 and Cdc2 in UM-UC-3 cells (Tang & Zhang, 2004). Singh and co-workers (Xiao et al. 2003, 2004; Singh SV et al. 2004) have shown that ITC, including AITC, PEITC and SF, down regulate Cdc25C in PC-3 cells, in addition to down-regulation of cyclin B1 and Cdc2 (AITC and PEITC) or cyclin B1 (SF). The level of Cdc25B, the function of which may be similar to that of Cdc25C, is also reduced in AITC- and SF-treated PC-3 cells. While it is not clear how the ITC down regulate cyclin B1 and Cdc2, ITC-induced down-regulation of Cdc25C appears to result, at least in part, from its phosphorylation at Ser-216 by checkpoint kinase 2 and translocation of phosphorylated Cdc25C from the nucleus to the cytoplasm as a result of increased binding with 14-3-3 protein and subsequent proteasome-mediated degradation (Singh SV et al. 2004; Xiao et al. 2004).

Both SF and AITC have been shown to arrest cells in the M phase, in addition to causing G2-phase arrest. SF-induced M-phase arrest is observed in murine mammary cancer F3IIl cells, human breast cancer MCF-7 cells and human pancreatic cancer MIA PaCa-2 cells and PANC-1 cells (Jackson & Singletary 2004a,b; Pham et al. 2004). AITC has a similar effect on human colon cancer HT-29 cells (Smith et al. 2004). These results therefore suggest that M-phase arrest is neither cell nor ITC specific. Both AITC and SF apparently target tubulin, disrupting intracellular tubulin
polymerization and spindle assembly, thereby inhibiting mitosis (Fig. 1).

Summary

Dietary ITC demonstrate potent anti-proliferative activity in cultured cell lines and animal models and their ability to attack multiple survival-related targets and signaling pathways. However, further study is needed to fully understand the anti-proliferative mechanism. Moreover, the vast and accumulating preclinical data make it possible to design and carry out human studies to evaluate the efficacy of dietary ITC in cancer prevention and treatment, which may be facilitated by the use of plants and plant extracts that are rich in ITC.

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