Anti-angiogenic effects of dietary isothiocyanates; mechanisms of action and implications for human health

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Anti-angiogenic effects of dietary isothiocyanates; mechanisms of action and implications for human health

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Running title; Isothiocyanates and angiogenesis

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Abbreviations;

4E-BP1, translation factor 4E binding protein 1; AITC, allyl isothiocyanate; Ang2, angiopoietin 2; AP1, activator protein 1; bFGF, basic fibroblast-like growth factor; BITC, benzyl isothiocyanate; BNIP3, BCL2/adenovirus E1B 19kDa interacting protein; CAIX, carbonic anhydrase IX; CBP, CREB binding protein; eIF4A, eukaryotic translation initiation factor 4A ; eIF4E, eukaryotic translation initiation factor 4E; eIF4F, eukaryotic translation initiation factor 4F; eIF4G, eukaryotic translation initiation factor 4G ; FIH, factor inhibiting HIF1; GLUT1, glucose transporter type 1 ; GSH, glutathione; GST, glutathione-*S*-transferases; HDAC, histone deacetylases; HIF, hypoxia inducible factor; HRE, hypoxia-response elements; HUVEC, human umbilical vein endothelial cells; IL8, interleukin 8; ITC, isothiocyanate; JNK, Jun N-terminal kinase; Keap1, kelch-like ECH-associated protein 1; mTOR, mammalian target of rapamycin; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κB ; PHD, prolyl hydoxylases; PEITC, phenethyl isothiocyanate; PGK1, phosphoglycerate kinase 1; ROS, reactive oxygen species; SFN, sulforaphane ; TGFβ, transforming growth factor β; VEGF, vascular endothelial growth factor; UTR, untranslated region; VHL, von Hippel-Landau protein

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Abstract

Isothiocyanates (ITCs) are electrophilic compounds derived from plants and are thought to play a major role in the potential chemopreventive effects associated with high intake of cruciferous vegetables. ITCs are also being evaluated for chemotherapeutic activity in early phase clinical trials. In addition to their effects on carcinogen metabolism and cancer cell survival and proliferation, ITCs have been shown to effectively interfere with angiogenesis in vitro and in vivo. Angiogenesis is the development of a new blood supply from existing vasculature and is required for tumours to develop beyond a small size limit determined by the diffusion limit for oxygen. Inhibition of angiogenesis may play a key role in the potential chemopreventive/chemotherapeutic activity of ITCs. In this review we highlight recent data demonstrating that ITCs have anti-angiogenic activity and identify potential molecular targets for these effects, including hypoxia-inducible factor (HIF), nuclear factor κB (NF-κB), activator protein 1 (AP1) and tubulin. We also discuss these findings in light of the potential chemopreventive/chemotherapeutic effects of ITCs.

Key words; isothiocyanates; angiogenesis; hypoxia inducible factor; nuclear factor κB; tubulin

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1. Introduction

Isothiocyantes (ITCs) are low molecular weight organic compounds with the general formula R-NCS. They are derived from plants where they are stored as glucosinolate precursors. Tissue damage due to chopping or mastication activates the plant enzyme myrosinase which hydrolyses the glucosinolate resulting in the formation of ITCs (Fig. 1). Variation in the R-group of glucosinolates gives rise to various ITCs which may exert distinct biological activities. Cruciferous vegetables are a particularly abundant source of ITCs and these compounds are thought to play a major role in the potential chemopreventive effects of diets rich in cruciferous vegetables. The anti-cancer activity of non-dietary administration of ITCs is also being evaluated in several early phase clinical trials.

The mechanisms by which ITCs exert their potential chemopreventive effects are likely to be multifactorial and are summarized in Table 1. For recent reviews please see [1-3]. The ability of ITCs to reduce carcinogen-induced tumourigenesis in experimental animals is relatively well understood; ITCs increase carcinogen detoxification via inhibition of carcinogen-activating phase I enzymes and induction of carcinogen-detoxifying phase II enzymes. However, more recently it has become clear that ITCs can also exert “direct” anti-cancer effects against established cancer cells in systems where carcinogens are not thought to play a role. For example, ITCs decrease in vivo growth of xenograft, as well as radiation- or genetically-induced, tumours. ITCs induce cell cycle arrest and cell death in cancer cells *in vitro*, and interfere with cancer cell invasion and metastasis (for recent reviews, see [1, 3, 4]). Importantly, normal cells appear to be relatively resistant to these effects. ITCs have recently been shown also to interfere with angiogenesis, a key process required for the progression of early tumours and a well established target for both chemopreventive and chemotherapeutic agents. In this review, we discuss the evidence that ITCs can act as anti-angiogenic agents. We also identify potential molecular mechanisms that mediate these effects and discuss these findings in light of the potential chemopreventive/chemotherapeutic effects of ITCs.

2. Chemistry and metabolism of ITCs

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The central carbon atom of the –NCS group is highly electrophilic and it is this reactivity with cellular nucleophiles that underpins the molecular effects and the metabolism of ITCs. Differences in the side-chain may modify the reactivity of ITCs by influencing electophilicity of the –NCS group, accessibility to nucleophilic centres and lipophilicity.

Following absorption, ITCs are predominantly metabolised by the mercapturic acid pathway. Conjugation of ITCs to glutathione (GSH) results in the formation of an ITC-GSH conjugate in a reaction catalysed by GST enzymes. ITC-GSH is subsequently converted to the cysteinylglycine conjugate by the action of γ-glutamyltranspeptidase which is then converted to the cysteine conjugate by the action of cysteinylglycinase. Finally, the ITC-cysteine conjugates are converted to *N*-acetylcysteine (NAC) conjugates by the action of *N*-acetyltransferase and excreted in the urine. The urinary excretion of ITC conjugates has been used to study the bioavailability of ITC, which appears to be quite high [5]. For example, when benzyl isothiocyanate (BITC) was administered orally to humans, 54% of the dose was recovered in the urine as BITC-NAC [6]. However, availability of ITCs from specific foods is influenced by preparation (i.e., raw versus cooked) and eating habit (i.e. extent of chewing) [7, 8].

Interestingly, polymorphisms within the genes encoding GST enzymes may influence the metabolism of specific ITCs and their potential chemopreventive effects. For example, Brennan et al. demonstrated that the protective effect of high cruciferous vegetable intake on lung cancer risk was greatest in those individuals with *GSTM1* and/or *GSTT1* null genotypes [9]. One possible explanation for these findings is that the presence of *GST* null genotypes decreases ITC metabolism and hence increases exposure. However, studies have shown that the *GSTM1* null genotype is associated with a greater rate of excretion of SFN metabolites [10] and other epidemiological studies have concluded that *GSTM1* positive individuals gain greater cancer protection from cruciferous vegetable ingestion than do *GSTM1* null individuals [11]. Since the effect of GST polymorphisms may be influenced by the predominant ITC consumed, there are likely to be complex interactions between ITC ingestion, genomics, and potential chemopreventive benefits.

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A particularly interesting feature of the pharmacokinetics of ITCs is their rapid and profound intracellular accumulation (Fig. 2). As discussed above, ITCs are rapidly conjugated to GSH, the most abundant cellular thiol (typically present at concentrations of 1 – 10 mM) via the action of GST enzymes following diffusion into cells. The resulting intracellular dithiocarbamates are exported from the cell by transporters such as multi-drug resistance protein-1 and p-glycoprotein-1 [12]. However, extracellular hydrolysis of GSH-ITC conjugates can also release free ITCs which are able to re-enter the cell where they are then able to react again with GSH. The net effect of this is a depletion of intracellular GSH and a large accumulation (100-200 fold over the extracellular ITC concentration) of intracellular ITCs. For example, treatment of Hepa 1c1c7 murine hepatoma cells with 100 μM SFN for 30 minutes resulted in total levels of intracellular SFN/SFN conjugates of 6.4 mM, 95% of which was SFN-GSH [13].

The consequences of this complex interplay between ITC conjugation, metabolism and efflux/uptake of ITCs are three-fold. First, GSH is a major antioxidant and its depletion leads to an increase in intracellular reactive oxygen species (ROS) [14]. Second, conjugates of ITCs generated via mercapturic acid pathway can themselves exert effects, for example, by the inhibition of histone deacetylases (HDACs) [15]. Finally, ITCs can react directly with other extracellular and intracellular targets. ITCs readily conjugate to protein cysteinyl thiols, whereas binding to DNA or RNA is not observed [16, 17]. For example, in phenethyl isothiocyante (PEITC)-treated A549 cells, intracellular levels of PEITC-GSH conjugates reached a maximum at 30 minutes post treatment and dropped rapidly thereafter. By contrast, PEITC-protein content gradually increased, accounting for 87% of total cellular content by 4 hours. Interestingly, sulforaphane (SFN)-protein conjugates accounted for only 12% of total SFN uptake at the same time point. Protein thiocarbamoylation can be a powerful modulator of protein function and is, for example, considered to play a key role in the induction of phase II carcinogen-metabolising enzymes by ITCs via induction of the Nrf2 transcription factor [18, 19]. PEITC appears to bind to proteins more readily than SFN, perhaps accounting for its more potent apoptosis promoting activity [Mi

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2007]. All three of these effects – increased ROS, direct action of metabolites and protein conjugation - are thought to contribute to anti-cancer effects of ITCs.

3. Tumour angiogenesis

Angiogenesis is the development of a new blood supply from an existing vasculature. The acquisition of angiogenic potential is considered a hallmark of malignant cells since it is required for tumours to grow beyond a small size (~1-2 mm in diameter), and to invade surrounding tissues and metastasise. Angiogenesis increases with cancer progression and has been linked to poor prognosis in a range of malignancies. Increased angiogenesis has also been observed in preneoplastic conditions [20], indicating that it plays a key role early in the process of carcinogenesis. Angiogenesis is a validated target for the development of both chemotherapeutic and chemopreventive agents.

Tumour angiogenesis is mediated by a complex interplay between malignant cells and endothelial cells, as well as stromal and immune cells and the extracellular matrix [21]. Cancer cells can produce a range of pro-angiogenic factors, including basic fibroblast-like growth factor (bFGF), vascular endothelial growth factor (VEGF), angiopoietin 2 (Ang2), interleukin 8 (IL8) and transforming growth factor β (TGFβ). These factors act to promote the survival, recruitment and proliferation of endothelial cells, resulting in the formation of new blood vessels. It is widely accepted that an "angiogenic switch" is activated when these pro-angiogenic factors outweigh the production of natural, anti-angiogenic factors (such as angiostatin, endostatin and thrombospondins). This switch can be triggered by immune/inflammatory responses, growth factors and genetic changes within cancer cells. However, hypoxia itself (i.e., low oxygen concentrations) is a major inducer of angiogenesis. Thus, cells which are located at a distance greater than the diffusion limit for O_2 (~100 μm) are hypoxic, leading to induction of pro-angiogenic factors and activation of the angiogenic switch.

4. Anti-angiogenic effects of isothiocyanates

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Various studies have shown that ITCs can interfere with angiogenesis in vitro and in vivo. Cultured human umbilical vein endothelial cells (HUVECs) are a well studied in vitro system to investigate modulation of angiogenesis and SFN has been shown to suppress both the proliferation and the matrigel-dependent tube forming ability of these cells [22]. Tube forming assays measure a complex series of events involving changes in endothelial cell morphology and migration, leading to the formation of a complex interconnecting network of capillary tubes with identifiable lumens (branching morphogenesis). Similar results have been shown for SFN using the immortalised human microvascular endothelial cell line, HMEC-1, and SFN was additionally demonstrated to decrease endothelial cell migration in a "wound healing assay" in these cells [23]. Similarly, PEITC has been shown to suppress HUVEC survival, migration (modified Boyden chamber) and tube formation [24] and allyl isothiocyanate (AITC) suppressed HUVEC proliferation, migration, invasion and tube formation [25]. In cultured bovine aortic endothelial cells, SFN-induced inhibition of proliferation was associated with G2/M phase cell cycle arrest [26].

In *ex vivo* tissue culture models, SFN caused a dose dependent reduction of microcapillary density in a placental vessel fragment outgrowth assay [23] and PEITC decreased vascular density in chicken egg chorioallantoic membrane assays [24]. AITC also decreased outgrowth of microvessels from rat aortic rings [27]. Intravenous administration of SFN decreased *in vivo* angiogenesis in VEGF-impregnated matrigel plugs [26] and intraperitoneal administration of AITC decreased *in vivo* capillary formation using the mouse B16F-10 melanoma model [27]. In these *in vivo* studies, the anti-angiogenic activity of ITCs was associated with reduced production of pro-angiogenic factors *in vivo*, including VEGF, nitric oxide and tumour necrosis factor α [25, 27]. SFN has been shown to reduce blood vessel density in MIA-PaCa2 xenografts *in vivo* [28]. Thus, various ITCs have been shown to exert anti-angiogenic activity in both *in vitro* and *in vivo* assays.

5. Modulation of hypoxia inducible factor activity

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Hypoxia inducible factors (HIF) may be key targets mediating the anti-angiogenic activity of ITCs. HIFs comprise a small family of dimeric basic helix-loop-helix transcription factors that play a central role in angiogenesis, linking hypoxia to the regulation of pro-angiogenic gene expression (e.g., VEGF), as well as regulators of cell survival (e.g. BCL2/adenovirus E1B 19kDa interacting protein 3 [BNIP3]), metabolism (e.g. glucose transporter type 1 [GLUT1], phosphoglycerate kinase 1 [PGK1]) and pH control (carbonic anhydrase IX [CAIX]) [29, 30]. The HIF family comprises three dimeric complexes, HIF1, 2 and 3, each of which comprises a hypoxia inducible alpha subunit (HIF1α, HIF2α or HIF3α) complexed with the constitutively expressed HIF1β subunit. The HIF1 and HIF2 factors are well characterised positive regulators of angiogenesis, whereas the function of HIF3 is less well understood.

The expression of HIFα subunits is tightly regulated via ubiquitylation and proteolysis [29, 30]. In normoxic conditions, HIFα subunits are hydroxylated on conserved proline residues by the action of oxygen sensitive prolyl hydoxylases (PHD) (Fig. 3). This triggers binding to the von Hippel-Landau (VHL) protein, an ubiquitin E3 ligase, and subsequent ubiquitylation and degradation via the proteasome. In hypoxic conditions, PHD activity is inhibited and HIFα-subunits are stabilized. Once HIFα-subunits accumulate, they associate with HIF1β and activate transcription by binding to hypoxia response elements in the promoters of target genes. The transactivating activity of HIF1α is also regulated by hypoxia. In normoxia, factor inhibiting HIF1 (FIH) hydroxylates asparagine residues within the C-terminal transactivation domain of HIF1α thereby displacing p300/CREB binding protein (CBP) coactivator proteins. Similar to PHD enzymes, FIH is oxygen sensitive; asparagine hydroxylation is decreased in hypoxia enabling p300/CBP binding and enhancement of transcriptional activation.

Several studies have demonstrated that ITCs modulate the expression and function of HIF, in both malignant and endothelial cells. Studies from our own laboratory have shown that PEITC effectively interferes with hypoxia-induced HIF transcriptional activity and the induction of the endogenous HIF target genes VEGF-A, BNIP3, CAIX and GLUT1 in human MCF7 breast cancer cells [31]. Similarly, SFN decreases

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VEGF production in response to hypoxia in human Tca8113 tongue squamous cell carcinoma cells and human DU145 prostate cancer cells [32]. In these studies, inhibitory effects of the ITCs appeared to be due to decreased translation of HIF1α/HIF2α, rather than decreased transcription or enhanced protein turnover [31, 32]. For example, inhibition of HIF activity in MCF7 cells was not associated with changes in *HIF1α* RNA levels. PEITC also suppressed the constitutive expression of HIF target genes in normoxia in RCC4 renal carcinoma cells, which are deficient in VHL and therefore unable to degrade HIFα proteins via the canonical VHL pathway. Indeed, using ^{35}S-metabolic labelling in MCF7 cells we have now directly demonstrated that PEITC suppresses HIF1α translation (BEC and GP, unpublished data). SFN also induced downregulation of HIF1α expression and VEGF production in HMEC1 cells [23]. However, in these cells, decreased HIF1α protein expression was associated with reduced *HIF1α* RNA levels suggesting a predominant role for transcriptional modulation.

A key question is how ITCs decrease HIF1α translation [31, 32]. PEITC has previously been shown to modulate the phosphorylation and expression of the protein translation factor 4E binding protein 1 (4E-BP1) [33]. 4E-BP1 is a key negative regulator of the translation promoting complex eukaryotic translation initiation factor 4F (eIF4F) which comprises three subunits, the scaffold protein eukaryotic translation initiation factor 4G (eIF4G), the RNA-helicase eukaryotic translation initiation factor 4A (eIF4A) and the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E) [34]. The function of eIF4F is tightly regulated by 4E-BP family proteins, of which 4E-BP1 is the most prominent. 4E-BP1 competes with eIF4G for an overlapping binding site on eIF4E in a phosphorylation-dependent manner. Dephosphorylation of 4E-BP1 increases affinity for eIF4E and therefore inhibits eIF4F. PEITC decreased 4E-BP1 expression and phosphorylation on multiple sites (Thr^{37}/Thr^{46}, Thr^{70} and Ser^{65}) in various cancer cell lines, including HCT116 (colorectal), PC3 (prostate) and MCF7 (breast) cells [31, 33], and decreased CAP-dependent RNA translation. Notably, the translation of RNAs with highly structured 5' untranslated regions (UTRs) is particularly dependent on the activity of eIF4F [35] and would therefore be expected to be particularly sensitive to

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4E-BP1 levels/phosphorylation. Consistent with this, PEITC preferably inhibited CAP-dependent translation of RNAs with a high level of secondary structure [33].

Interestingly, the 5' UTR of the HIF1α mRNA is highly structured, suggesting it may be particularly sensitive to translation inhibition by ITCs. This sensitivity is apparent in cells treated with the immunosuppressant rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR)-containing mTORC1 complex, a major regulator of 4E-BP1 phosphorylation. Thus, mTORC1 inhibition leads to 4E-BP1 dephosphorylation and decreased translation of RNAs with highly structured 5'-UTRs, including HIF1α [36]. However, effects of PEITC on translation are likely to be complex. For example, we demonstrated that PEITC also decreased expression of HIF2α protein in MCF7 cells [31], although HIF2α RNA does not have a highly structured 5'-UTR and is not effectively repressed by rapamycin [36]. Thus, ITCs may affect global protein production via inhibition of CAP-dependent translation, although some RNAs with highly structured 5'-UTRs might be particularly susceptible. Importantly, overexpression of eIF4E protected HCT116 from PEITC-induced growth inhibition, indicating that translation repression contributes to the growth inhibitory effects of PEITC [33]. However, since these experiments were performed in normoxia, modulation of HIF activity is unlikely to be important for these effects. Enhanced translation of cell survival (Bcl-X_L) and proliferation (cyclin D1) proteins, known to be induced following eIF4E overexpression in other systems, might contribute to protection from PEITC-induced growth inhibition. Taken together, PEITC (and presumably other ITCs) may exert a general inhibitory effect on translation. However, HIF1α is highly sensitive to decreased translational efficiency and decreased HIF1α translation is likely to be an important mechanism mediating anti-angiogenic effects of ITCs.

6. HIF-independent control of angiogenesis

There are also HIF-independent pathways of tumour angiogenesis (Fig. 4) [37]. For example, knock-down of HIF1α does not prevent angiogenesis or VEGF expression in xenograft models of colon cancer [38]. The transcription factors nuclear factor kappB (NF-κB), activator protein-1 (AP1) and MYC activate expression of pro-

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angiogenic factors, such as VEGF and/or IL-8 [37], and inhibition of these factors by ITCs may also contribute to anti-angiogenic effects. RAS oncoproteins appear to act as master regulators of these HIF-independent pathways [37]. Given the importance of these HIF-independent pathways, it is important to consider whether their modulation might also contribute to anti-angiogenic effects of ITCs.

The NF-κB family of dimeric transcription factors are widely expressed and play a key role in controlling the onset and the resolution of inflammation as well as stress responses, intercellular signalling, cellular proliferation, angiogenesis and apoptosis [39]. In most cells, NF-κB proteins are complexed with inhibitor proteins (IκB proteins) in an inactive form in the cytoplasm. Various stimulators cause the activation of a multiple-protein kinase complex (IKK) which phosphorylates IκB proteins targeting them for degradation and leading to the nuclear translocation and activation of NF-κB. NF-κB is thought to play a key HIF-independent role in tumour angiogenesis, via induction of IL8 and VEGF. Indeed, increased production of ROS due to HIF inhibition activates NF-κB and heightens its role in angiogenesis [38]. Also, inactivation of PHDs in hypoxia can activate NF-κB following decreased hydroxylation of IKKβ [40].

The first reported study of ITC-mediated NF-κB inhibition demonstrated that SFN decreased NF-κB DNA binding activity and target gene expression in lipopolysaccharide treated RAW264.7 mouse macrophages [41]. Since then ITCs have been shown to decrease NF-κB activity in various human cancer cell lines, including HT-29 (colorectal), BxPC3, MIA-PaCa-2 (both pancreatic cancer), PC3 (prostate cancer) and T24 (bladder cancer) [28, 42-45]. Various mechanisms may contribute to modulation of NF-κB activity. For example, Heiss et al. demonstrated that SFN did not block IκBα/β degradation or nuclear import of the NF-κB subunits p50 (NF-κB1) and p65 (RelA), but could directly interfere with NF-κB DNA binding *in vitro* [41]. By contrast, others have shown that ITCs reduce phosphorylation and increase expression of IκBα [42-44, 46]. SFN and PEITC inhibited IKKβ-mediated phosphorylation of IκBα in an *in vitro* assay, suggesting that IKKβ may be a direct target for modulation by ITCs [44].

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As discussed above, protein thiocarbamoylation is thought to play an important role in the anticancer effects of ITCs [47]. For example, direct covalent modification of the negative regulator kelch-like ECH-associated protein 1 (Keap1) by SFN is thought to contribute to activation of Nrf2, required for activation of phase II carcinogen-inactivating enzymes and protection from carcinogen-induced cancer [18, 19, 48, 49]. Interestingly, a number of reactive chemical compounds have been shown to inhibit NF-κB activity via direct covalent modification of NF-κB signalling pathway components. For example, the DNA binding domains of the p50 and p65 NF-κB subunits contain redox-regulated cysteine residues (Cys^{62} and Cys^{38}, respectively) that have been shown to be covalently modified by andrographolide and picroliv, respectively, thereby preventing DNA binding [50, 51]. Also, IKKβ contains a redox regulated cysteine residue (Cys^{179}) that is essential for activity [52], and is modulated by other agents such as curcumin and butein (3,4,2,4-tetrahydroxychalcone) [53, 54]. Thus, these cysteine residues are candidate sites for thiocarbamoylation by ITCs, although direct evidence for covalent binding to NF-κB subunits by this class of molecule is lacking. Very recent studies have demonstrated that, in addition to Nrf2, Keap1 can function as a ubiquitin E3 ligase for IKKβ and thereby downregulate NF-κB activity [55]. However, it is not known whether modulation of Keap1 by ITCs *per se* leads to increased expression/activity of IKKβ.

The AP1 transcription factor is also important for angiogenesis. AP1 comprises a dimer of Jun/Fos family proteins that modulates specific gene expression in response to a wide range of stimuli, including growth factors, stress, infection and oncogenic stimuli [56, 57]. The AP1 activating kinase Jun N-terminal kinase (JNK) is activated downstream of K-RAS in hypoxic cells, and JunB may be required for NF-κB mediated induction of VEGF [58]. Inhibition of AP1 by ITCs has been observed in cancer lines, including HT29 cells, and in the skin *in vivo* following UVB-irradiation [59-61]. *In vitro* studies have shown that SFN-inhibition of AP1 DNA binding is dependent on Cys^{154} of c-Fos and Cys^{272} of c-Jun, suggesting that these may be sites of direct covalent modification by ITCs [61]. However, the effects of ITCs on AP1 are complex and other studies have reported induction of AP1 transcriptional activity by ITCs in a range of cell lines [59, 60, 62, 63] .

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The MYC oncoprotein also plays a key role in angiogenesis, by upregulation of VEGF and downmodulation of thrombospondins. MYC expression is induced in hypoxic cells via activation of PI3K→Rho→ROCK signalling. ITCs downmodulate MYC expression in HMEC1 cells [23] but the mechanism for this remains unknown.

7. Covalent modification of tubulin

Only a small number of direct targets for ITC-mediated thiocarbamoylation have been identified to date and the potential impact on NF-κB and AP1 have been discussed above. However, the best characterized target are tubulins. Tubulins are a family of globular proteins which assemble to form microtubules. Microtubules are essential for chromosome segregation during mitosis and inhibition of microtubule function may therefore be one mechanism leading to ITC-induced mitotic cell cycle arrest (including in endothelial cells [26]). However, tubulins also play important roles in angiogenesis in addition to proliferation of endothelial cells since they are also required for morphogenesis and migration.

The ability of SFN to inhibit tubulin polymerisation *in vitro* and in intact cells had been recognised for some time [64, 65]. However, radiolabelling experiments have now demonstrated that PEITC and SFN directly conjugate to both α- and β-tubulin isoforms [66]. Multiple molecules of ITCs interact with individual tubulin molecules, at least *in vitro*, and BITC has been shown to conjugate to Cys^{347} of α-tubulin in intact cells. BITC and PEITC effectively inhibit tubulin polymerisation *in vitro* and disrupt microtubules in intact cells, whereas SFN exerts more modest effects [66]. More recently it has been shown that BITC and PEITC, but not SFN, also promote the proteasome-dependent degradation of α/β tubulin in HeLa cells and non-small cell lung cancer-derived H460 cells [67] and PC3 and DU145 prostate cancer cells [68]. Pharmacological anti-tubulin agents can exert powerful antiangiogenic effects, so inhibition of tubulin could also contribute to inhibition of angiogenesis by ITCs. Interestingly, microtubule agents also inhibit the translation of *HIF1α* RNA through a poorly defined mechanism [69] and thus might also play a role in inhibition of HIF1α protein expression.

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8. A role for ROS and ITC metabolites?

In addition to protein thiocarbamoylation, the anti-cancer activities of ITCs are thought to involve increased production of ROS and effects of ITC metabolites. Although their effects are complex, ROS are generally considered to promote angiogenesis so it is unlikely that an initial production of ROS in ITC-treated cells would contribute to suppression of angiogenesis. However, it is important to bear in mind that although initially acting as pro-oxidants, ITCs can also act as indirect anti-oxidants. ITCs can lead to activation of Nrf2, a master inducer of anti-oxidant gene expression. Thus, following prolonged exposure to ITCs, raised intracellular anti-oxidant status may contribute to reduced angiogenesis. Metabolites of ITCs can act as inhibitors of HDACs [70]. HDACs and their counteracting partners, histone acetyltransferases, control the acetylation status of not just histones, but also a wide range of other cellular proteins, including HIF1. HIF1 transcriptional activity is inhibited by acetylation of HIF1α suggesting that inhibition of HDACs via ITC-metabolites might also impact on HIF activity. Indeed, pharmacological HDAC inhibitors have anti-angiogenic activity [71]. However, there is no evidence that HIF acetylation is modulated in ITC-treated cells.

9. Relevance for chemoprevention and chemotherapy

It is important to consider these results linking ITCs to inhibition of angiogenesis, as well as their effects on other cancer associated pathways (Table 1), in the context of their potential chemopreventive/chemotherapeutic activity. Cruciferous vegetables are a rich dietary source of ITCs and although recent prospective studies have suggested that the relationship between cancer risk and cruciferous vegetable intake is more complex than originally thought, there remains intense interest in the potential chemopreventive activities of ITCs. Given the key role of angiogenesis for the progression of early tumours (and potentially also pre-neoplastic lesions), inhibition of angiogenesis may play an important role in the potential chemopreventive effects of these compounds. Notably, “classical” anti-angiogenic

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drugs, such as the anti-VEGF antibody Bevacizumab typically extend patient survival by only a relatively short period. These limited clinical responses are perhaps not surprising given that these agents selectively target just one of many factors that act in concert to modulate angiogenesis. In this regard, the multifunctional effects of ITCs may be beneficial in providing an effective block to angiogenic activity.

The critical question is whether normal dietary intake of cruciferous vegetables might be adequate to deliver sufficient concentrations of ITCs to inhibit angiogenesis (or, indeed, other cancer promoting pathways that have been shown to be suppressed by ITCs *in vitro*). One approach to address this question is to compare the levels of ITCs that appear in the blood following ingestion of glucosinolate-rich foods with the concentrations required to exert effects *in vitro* (see Table 2 for a summary of molecular effects of ITCs potentially linked to anti-angiogenic activity with an indication of effective concentrations). In general, ITC concentrations required for *in vitro* modulation of angiogenesis, or to exert effects on molecular regulators such as HIF, are in the order of 1-20 μM, and only few studies report significant effects at concentrations <1 μM [23, 24]. Pharmacokinetic studies have demonstrated that ITCs are detectable in the blood following ingestion of cruciferous vegetables, but these levels would appear to fall short of those required for modulation of angiogenesis pathways. For example, in two studies, the mean peak plasma concentrations of PEITC following ingestion of 80 or 100 g of watercress were 297 and 928 nM, respectively [72, 73]. The mean peak plasma concentration of SFN following ingestion of 150 ml of broccoli "soup" equivalent to 100 g of broccoli was ~650 nM [74]. However, these comparisons are complicated by the presence of ITC-metabolites, some of which may be active. For example, whereas the peak plasma concentration of SFN following ingestion of 150 ml of broccoli "soup" was only ~650 nM, the total amount of SFN plus SFN metabolites (i.e. SFN-GSH, SFN-cysteine-glycine, SFN-N-acetylcysteine and SFN-cysteine) was ~2 μM and this rose to ~7 μM following ingestion of an equivalent amount of "super-broccoli", bred to provide higher levels of ITCs [10, 74]. Moreover, even partial inhibition of multiple molecular regulators that may be achieved with relatively low levels of ITCs may cooperate to provide effective suppression of angiogenesis.

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Another limitation of measuring plasma ITC levels is that it is the intracellular levels which are presumably of most importance. Intracellular concentrations of ITCs may vary from cell type to cell type due a complex interplay between metabolism, GSH-conjugation and transport (Fig. 2). Therefore, an alternate approach to assess the bioavailability of dietary ITCs is to measure effects on intracellular biomarkers in circulating blood cells. Rowland and colleagues have demonstrated reduced levels of basal and hydrogen peroxide-induced DNA damage in peripheral blood lymphocytes, presumably due to Nrf2-dependent increased antioxidant gene expression, following daily intake of 85 g of watercress for 8 weeks [75]. Our own pilot work has shown that ingestion of watercress (80 g) appears to be sufficient to cause a transient decrease in 4E-BP1 phosphorylation [73] mirroring effects seen *in vitro* in cancer cells [31, 33]. Moreover, ingestion of SFN-enriched broccoli sprouts is associated with inhibition of HDAC activity and accumulation of acetylated histone [76] consistent with the *in vitro* HDAC-inhibitory activity of ITC metabolites. Thus, although falling well short of proof that dietary intake of ITCs has anti-cancer effects, these experiments provide encouraging data that the bioavailability of ITCs is sufficient to modulate pathways which have been associated with anti-cancer effects of ITCs in *in vitro* studies. Further studies are clearly required but molecular biomarker analysis may be a powerful approach to address the *in vivo* effects of dietary intake of ITCs, including effects on angiogenesis.

The findings described here are also relevant in the context of clinical trials designed to explore the potential chemopreventive/chemotherapeutic effects of ITCs. We identified 4 clinical trials evaluating either pure PEITC or SFN-enriched broccoli sprout extract in cancer clinical trials (http://www.cancer.gov/clinicaltrials). PEITC induces apoptosis of primary chronic lymphocytic leukaemia cells *in vitro* [77] and a planned phase I trial (NCT00968461) will determine the maximum tolerated dose of oral PEITC in patients with previously treated lymphoproliferative disorders. A randomized and placebo-controlled phase II trial (NCT00691132) will assess the effects of oral PEITC in lung cancer in smokers. The primary objective of this trial is determine the effect of PEITC on metabolism of the cigarette smoke procarcinogen 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) whereas secondary objectives include histopathological evaluation of lung lesions and analysis of cell cycle and apoptosis biomarkers in bronchial tissue. A phase I trial (NCT00005883)

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will determine the tolerability and toxicity of oral intake of SFN-enriched broccoli sprout extract in patients with invasive transitional cell carcinoma of the bladder (with secondary aims to study the effects on tumour angiogenesis, cell proliferation and apoptosis). Finally, a randomised, placebo-controlled phase II trial (NCT00843167) effect will assess the effects of broccoli sprout extract on markers of apoptosis and cell proliferation, and on inhibition of HDACs, in women newly diagnosed with ductal breast carcinoma *in situ* and/or atypical ductal hyperplasia. The results described here suggest that clinical evaluation of ITCs could be extended to (pre)malignancies associated with pronounced angiogenesis, such as renal cell carcinoma. Although normal dietary intake of ITCs is widely considered to be safe, the same may not be true of pharmacological doses and an important goal of these trials will be to assess safety. Indeed, widespread inhibition of multiple pathways, as may be expected for pleiotropic agents such as ITCs, may lead to significant toxicity. It will also be important to consider the potential for drug-drug interactions since induction of drug efflux pumps and phase I and II drug metabolising enzymes by ITCs may alter the metabolism and disposition of other pharmacological agents [78]. For example, even intake of 50 g of watercress has been shown to alter metabolism of acetaminophen [79].

10. Conclusions

The studies described in this review provide compelling evidence that, in addition to their well characterized effects on carcinogen activation and cell cycle and apoptosis, ITCs can exert anti-angiogenic activity in *in vitro* and *in vivo* models. However, it remains unclear to what extent these effects contribute to the potential chemopreventive activity of dietary intake of ITCs. The mechanisms by which ITCs modulate angiogenesis are likely to be multifactorial and complex, which presumably reflects the pleiotropic effects of these compounds. HIF is a major regulator of angiogenesis and its inhibition, perhaps predominantly via suppression of protein translation, is likely to play a central role in the anti-angiogenic effects of ITCs. However, angiogenesis is also controlled independently of HIF, and key roles have been ascribed to other transcription factors, including NF-κB, AP1 and MYC, and to

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tubulin, all of which can be modulated by ITCs. Further studies are required to understand molecular mechanisms by which ITCs exert their anti-angiogenic effects, and whether normal dietary intake or pharmacological administration of ITCs is sufficient to modulate these pathways *in vivo*.

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Figure legends

Fig. 1. Isothiocyanates. Myrosinase-mediated generation of ITCs (R-NCS) from glucosinolate precursors (top) and structures of representative dietary ITCs (bottom).

Fig. 2. Intracellular accumulation of ITCs. Following their initial diffusion into cells (1), ITCs are rapidly conjugated to glutathione (GSH) via the action of glutathione-S-transferases (GST) (2). The GSH conjugates are exported from the cells via efflux pumps (3). Extracellular hydrolysis of the GSH conjugate (4) gives rise to the free ITC which is able to re-enter the cell. The net effect of this cycle is the rapid (1-3 hr) and profound (100-200 fold) accumulation of ITC and the concomitant depletion of GSH. Once GSH levels are reduced, ITCs are able to conjugate to intracellular protein cysteinyl thiols.

Fig. 3. Regulation of HIF1 activity. In normoxia, hypoxia-inducible factor (HIF)-1α is hydroxylated by proline hydroxylases (PHD) in the presence of O_2. Hydroxylated HIF-1α is recognised by VHL (von Hippel–Lindau protein) resulting in polyubiquitination and proteasomal degradation. In response to hypoxia, proline hydroxylation is inhibited. VHL is no longer able to bind and target HIF-1α for proteasomal degradation and HIF-1α accumulates and translocates to the nucleus. HIF-1α dimerises with HIF-1β, binds to hypoxia-response elements (HREs) within the promoters of target genes and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity. CBP, CREB-binding protein; Ub, ubiquitin. Adapted from Ref. [30].

Fig. 4. HIF-dependent and independent regulation of angiogenesis. Activation of HIF by inhibition of prolyl hydroxylase activity in hypoxic conditions plays a key role in induction of pro-angiogenic factors, including interleukin 8 (IL8), angiopoietin 2 (Ang2) and vascular endothelial growth factor (VEGF). HIF1 activity is also highly dependent on efficient translation of the *HIF1α* RNA. However, NF-κB, AP1 and MYC also play key HIF-independent roles via induction of pro-angiogenic factors and repression of anti-angiogenic factors (e.g., thrombospondin-1, Tsp1). RAS can be activated by hypoxia and is a master regulator of these HIF-independent pathways.

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HIF depletion leads to enhanced production of reactive species, leading to activation of NF-κB and induction of IL8. Potential targets for the anti-angiogenic effects of ITCs are shown in boxes along with specific ITCs that have been shown to modulate these targets – see Table 2 and text for details. Solid and dashed lines indicate positive and negative regulation, respectively and in some cases this involves intermediate regulators which are not shown. Adapted from Ref. [37].

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Table 1. Potential mechanisms contributing to anti-cancer effects of ITCs.
[a]Typical effective concentrations. Note that differences in assay design preclude direct comparison between studies.
In the interests of space, only selected references provided.

Biological response	**Effect**	**Conc[a]**
Regulation of P450 enzymes	ITCs inhibit or downregulate various cytochrome P450 enzymes, important for activation of chemical carcinogens	13 μM (BITC) [80] 1.8 μM (PEITC) [81] 180 nM, 340 nM (PEITC) [82] 1 μM (PEITC) [83] 10 μM (SFN) [84]
Induction of antioxidant gene expression	ITCs increase activity of Nrf2 a key transcription factor involved in induction of oxidant/electrophile response genes and mediators of chemopreventive activity	7.5 μM (PEITC) [85] 25 μM (SFN, AITC) [86] 0.5 μM (PEITC) [87]
Cell cycle arrest	Inhibition of cell cycle progression has been observed in a wide variety of cell lines derived from both solid and haematological malignancies. Arrest in G_1, S or G_2/M phases of the cell cycle has been described.	10 μM (AITC), 10 μM (BITC) [88] 2.5 μM (BITC) [89] 10 μM (SFN) [90] 12 μM (AITC) [65] 15 μM (SFN) [91] 5 μM (PEITC) [92] 5 μM (BITC) [43]
Induction of apoptosis	Induction of both the intrinsic and extrinsic apoptosis pathways has been observed in a wide variety of cell lines derived from both solid and haematological malignancies.	5 μM (PEITC) [93] 10 μM (PEITC) [94] 5 μM (SFN) [90] 15 μM (SFN) [91] 5 μM (BITC) [43] 10 μM (PEITC) [95]
Induction of	Appears to require higher concentrations	25 μM (PEITC/BITC)

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necrosis	than induction of apoptosis	[96] 50 μm (BITC) [97]
Metastasis	ITCs inhibit cell adhesion, invasion and migration in vitro, and metastasis in vivo	5 μM (AITC) [98] 5 μM (PEITC) [99] 2 μM (PEITC) [24] 2 μg/ml (SFN) [100]
Inhibition of angiogenesis	ITCs inhibit angiogenesis in in vitro and in vivo models	1 μM (PEITC) [24] 5 μg/ml (AITC) [27] 0.1 – 1 μM (SFN) [23]

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Table 2. Summary of in vitro effects of ITCs on potential molecular targets mediating anti-angiogenic effects.
[a]effective concentrations. Note that differences in assay design preclude direct comparison between studies.

Target	Effect	Conc (μM)[a]	Refs
HIF	SFN decreased HIF1α RNA levels in HMEC-1 cells.	6.25	[23]
	PEITC inhibited HIF transcriptional activity in MCF7 cells. Associated with reduced HIF1α protein expression.	3.25	[31]
	SFN reduced HIF1α protein expression in Tca8113 and DU145 cells	5 – 10	[32]
MYC	SFN decreased *MYC* RNA levels in HMEC-1 cells.	1.56	[23]
NF-κB	SFN inhibited NF-κB DNA binding activity in lipopolysaccharide-stimulated RAW264.7 macrophages, independent of effects on IκB. Mechanism may involve direct inhibition of DNA binding and/or modulation of thioredoxin reductase.	10	[41] [101]
	BITC inhibited NF-κB DNA binding activity in Bx-PC3 cells, associated with reduced IκBα phosphorylation.	20	[43]
	SFN inhibited NF-κB DNA binding activity in human pancreatic adenocarcinoma cells.	10	[28]
	PEITC/SFN inhibited NF-κB transcriptional activity in PC-3 cells. Associated with decreased phosphorylation of IKKα and IKKβ.	5 (PEITC) and 20 (SFN)	[44]
	PEITC, SFN, AITC inhibited LPS-induced	10	[42]

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	NF-κB transcriptional activity in HT-29 cells	(PEITC), 10 (SFN) and 25 AITC	
	SFN decreased TNFα-induced NF-κB activity in human leukaemia cells, associated with reduced IκBα phosphorylation	1	[46]
AP1	PEITC decreased TPA-induced AP1 transcriptional activity in HT-29 cells at high concentrations	35	[59]
	SFN inhibited UVB-induced AP1 transcriptional activity in HCL14 cells.	10	[60]
Tubulin	BITC, PEITC and SFN disrupted microtubules in A549 cells	5 (BITC), 5 (PEITC) and 10 (SFN)	[66]
	BITC and PEITC triggered proteolysis of α- and β-tubulin in A549 cells	10 (BITC) and 10 (PEITC)	[66]

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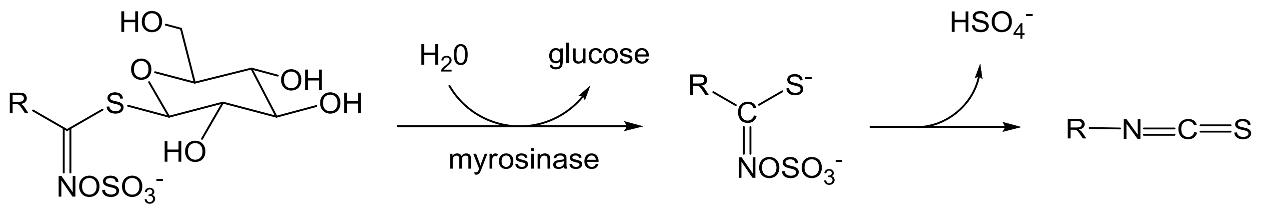
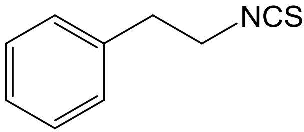
Figure 1





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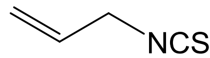

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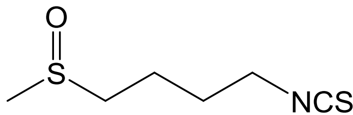
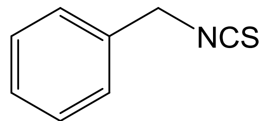


sulforaphane (SFN)



benzyl isothiocyanate (BITC)

Figure 2

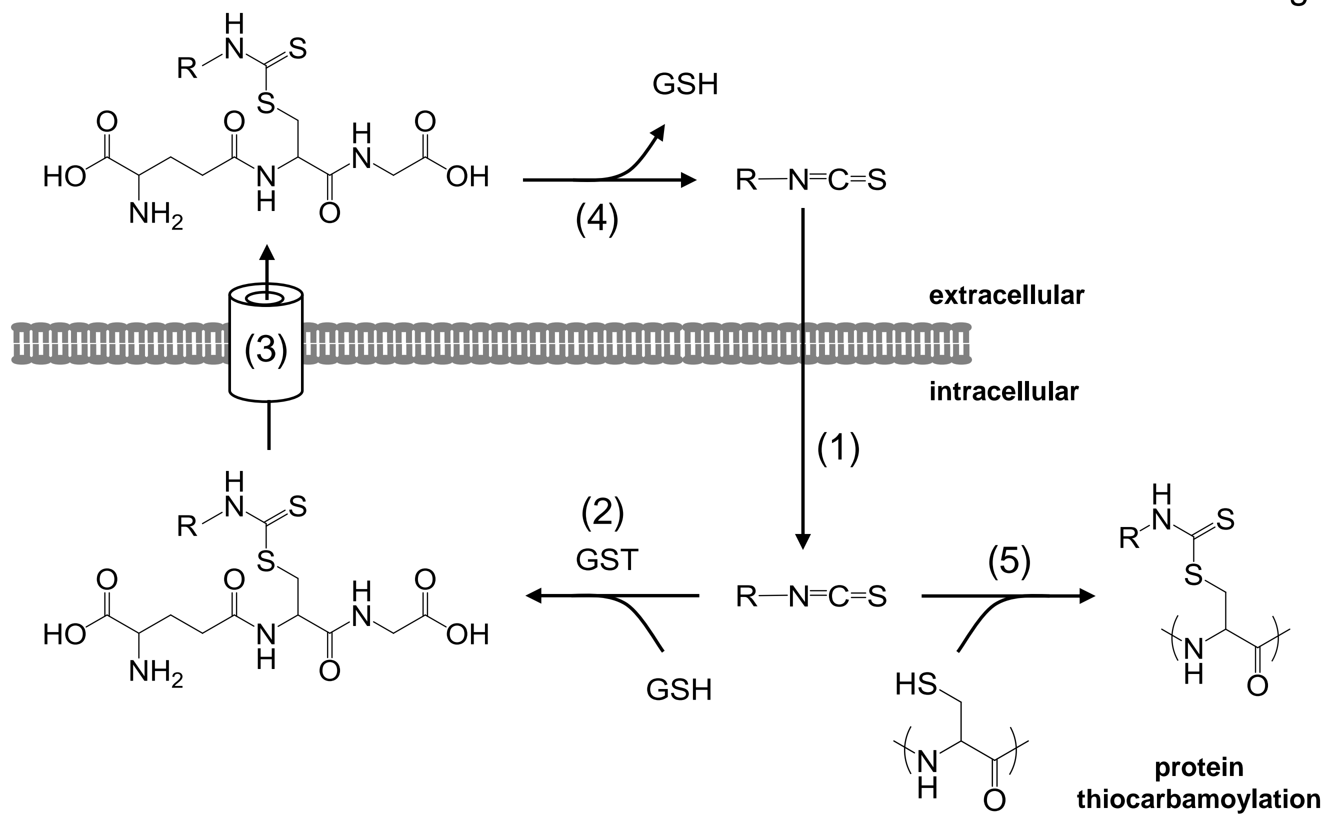


Figure 3 B&W

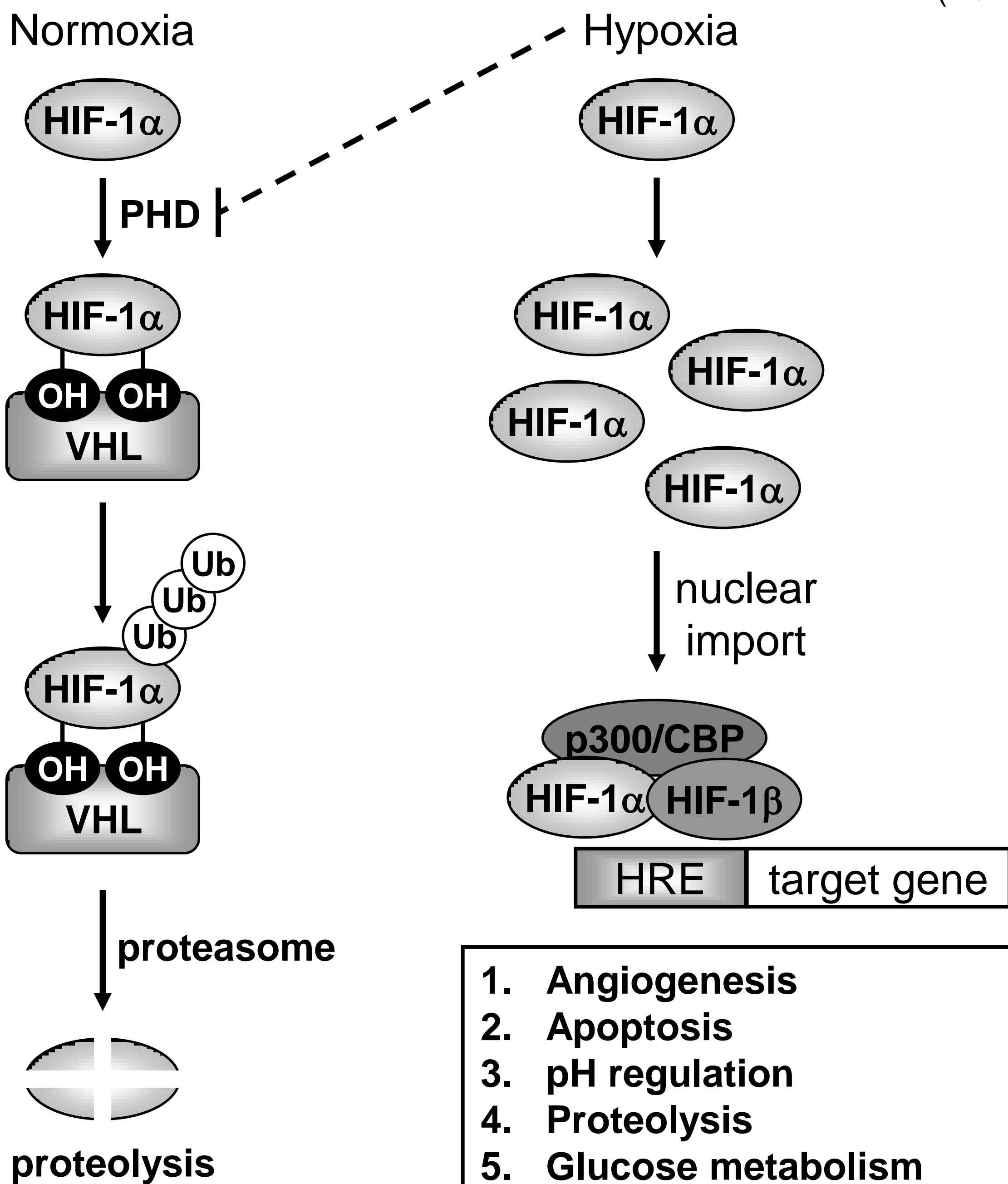


Figure 3 colour

Figure 3 (colour on-line only)

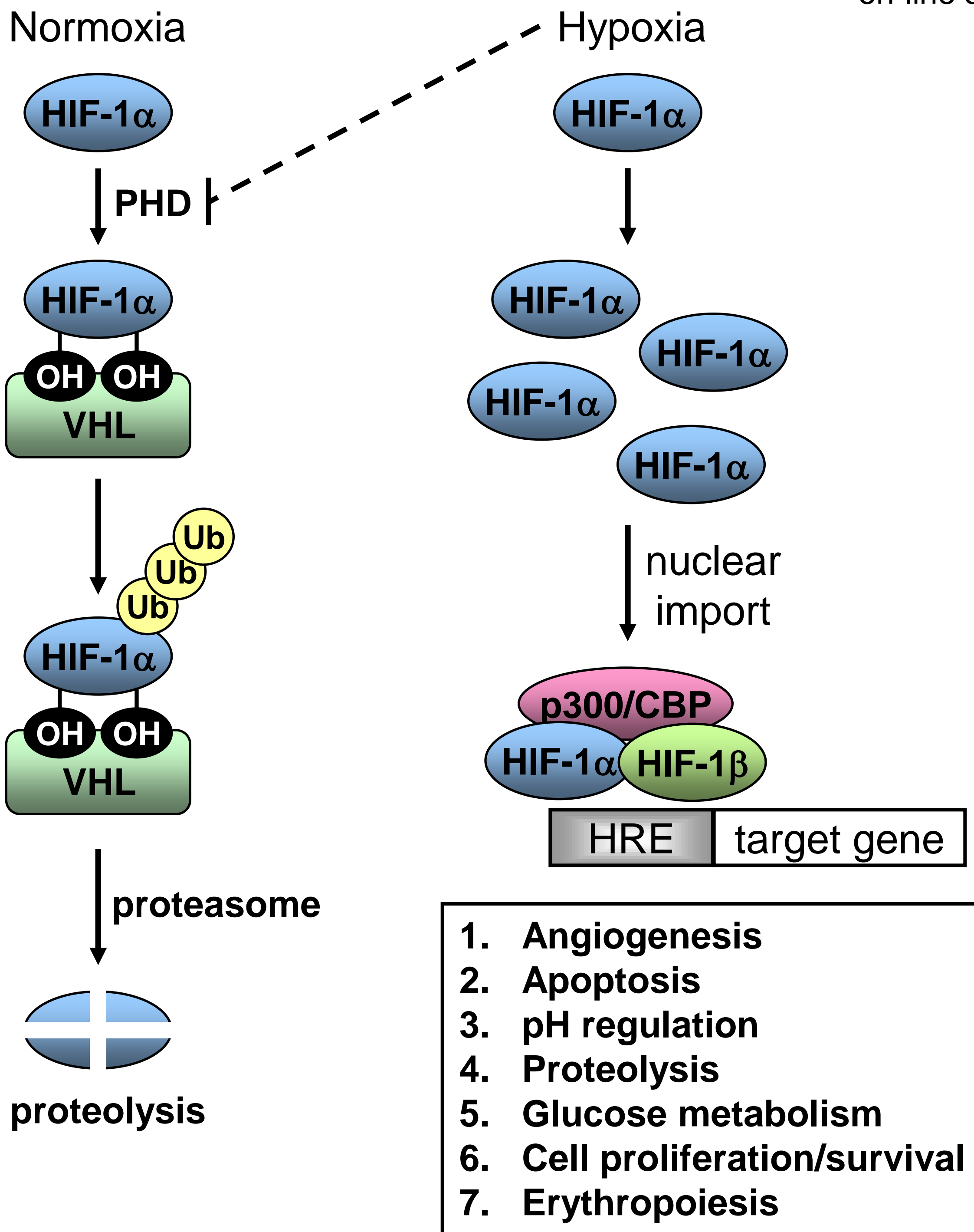


Figure 4 B&W

Figure 4
(B&W)

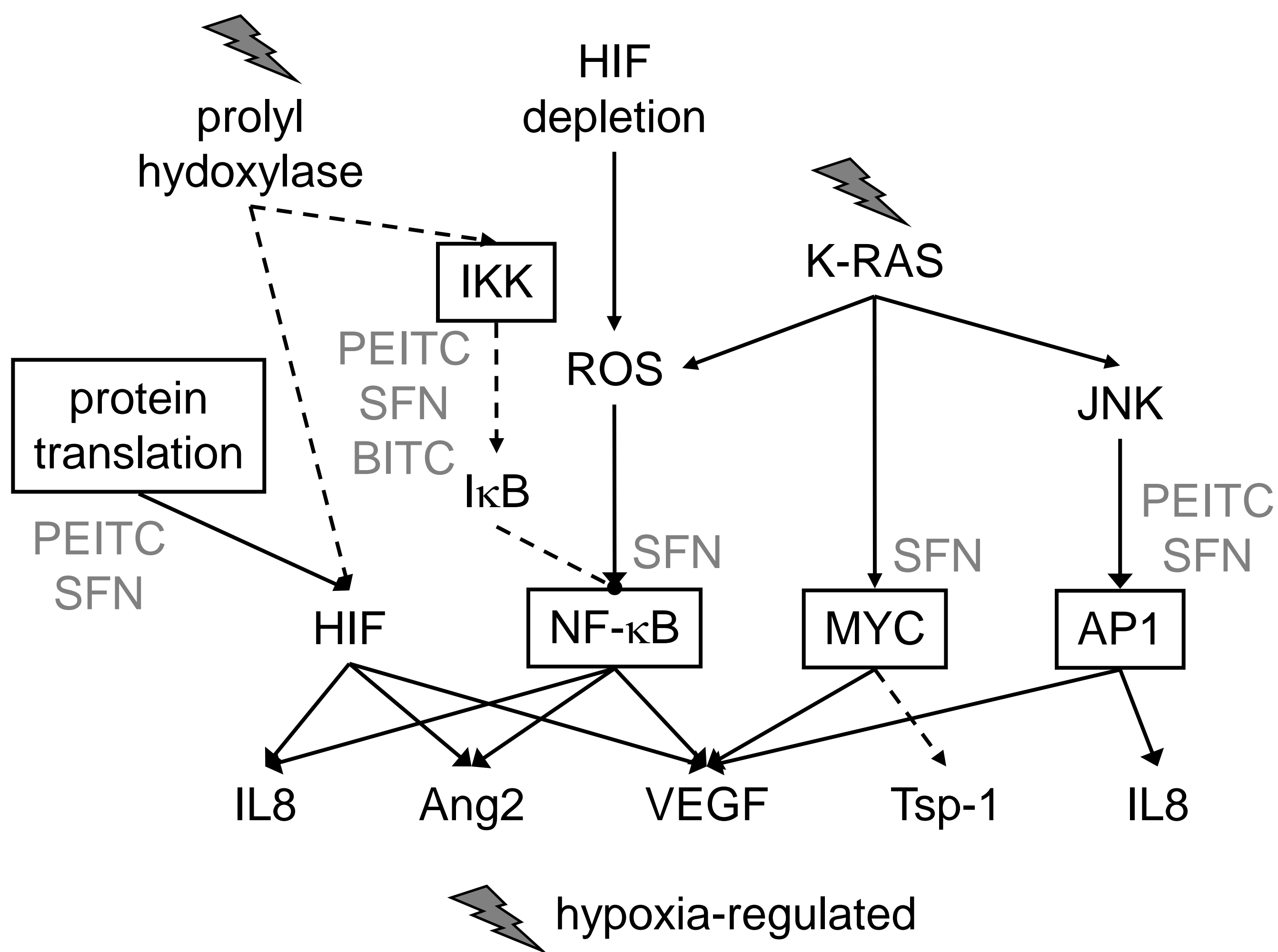
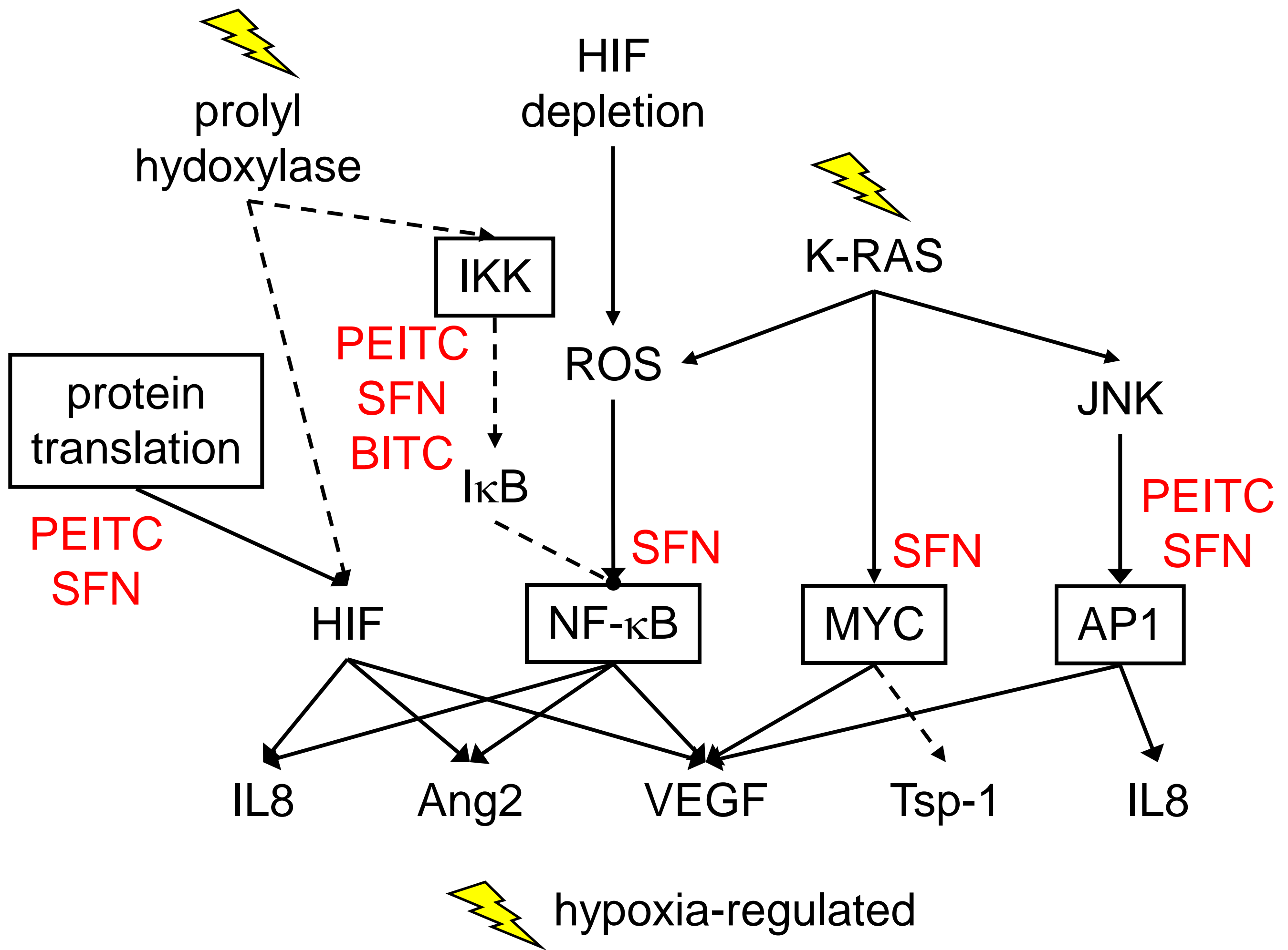


Figure 4 colour

Figure 4
(Colour
on-line only)



*Graphical Abstract