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Chemopreventive functions of sulforaphane: A potent inducer of antioxidant enzymes and apoptosis

Chi-Tai Yeh[a], *Gow-Chin Yen*[b,*]

[a]*National Institute of Cancer Research, National Health Research Institutes, Miaoli County 350, Taiwan*
[b]*Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan*

ARTICLE INFO

Article history:
Received 30 July 2008
Accepted 22 August 2008
Available online 11 October 2008

Keywords:
Chemopreventive agent
Sulforaphane
Signaling pathway
Metallothionein
Apoptosis

ABSTRACT

Epidemiological and dietary studies have revealed an association between high intake of cruciferous vegetables and decreased cancer risk cancer. Sulforaphane, a phytochemical constituent of cruciferous vegetables, has received much attention as a potential cancer chemopreventive compound. Recent advances in the cellular and molecular biology of cancer have shed light on components of intracellular signaling cascades that can be molecular targets of chemoprevention by various anti-cancer agents. Metallothionein (MT), a primary antioxidant enzyme involved in the metabolism and detoxification of heavy metal, has been recognized as a molecular target for chemoprevention by natural anti-cancer agents, but the cellular signaling mechanisms that associate MT gene regulation are not yet clearly understood. Recent studies suggest that Nrf2-mediated signaling, which controls the expression of many of genes responsible for carcinogen detoxification and protection against oxidative stress, is regulated by sulforaphane. This contribution focuses on Nrf2-mediated signaling pathways, particularly in relation to MT gene induction and the apoptosis-inducing effects of sulforaphane.

1. Introduction

Carcinogenesis can be viewed as a process that involves abnormal cellular changes in which the genes controlling proliferation, differentiation, and apoptosis are transformed under selective environmental pressures (Blagosklonny, 2005). Tumor development follows three distinct phases: initiation, promotion, and progression (Krishnan et al., 2003). The initiation phase is a rapid and irreversible process that occurs when normal cells are exposed to a carcinogen that causes irreparable or mis-repaired DNA damage. DNA damage itself is not carcinogenic unless the resulting somatic mutation is propagated via mitosis to yield a clonal population of mutated cells. In contrast to initiation, tumor promotion is a relatively lengthy and reversible process involving the accumulation of actively proliferating preneoplastic cells. Progression, the final stage of neoplastic transformation, involves the growth of a tumor with invasive and metastatic potential. One rational and effective approach to controlling cancer is the inhibition, reversal or delay of genetic and epigenetic events that result in the neoplastic transformation of cells (Stoner et al., 1997).

The term 'chemoprevention', first coined by Michael Sporn in the mid-1970s, refers to the use of non-toxic substances, including many food factors, to interfere with the process of cancer development or carcinogenesis before invasion and metastasis can occur (Smith et al., 2005). Chemoprevention has successfully been achieved in numerous *in vitro* as well as *in vivo* studies over the past 25 years, and has been validated in several human intervention trials (Sporn & Liby,

* *Corresponding author:* Tel.: +886 4 22879755; fax: +886 4 22854378.
E-mail address: gcyen@nchu.edu.tw (G.-C. Yen).
1756-4646/$ - see front matter
doi:10.1016/j.jff.2008.09.002

2005). One important process in chemoprevention involves modulation of the activity of phase II antioxidant enzymes, which convert carcinogens to inactive metabolites that are readily excreted from the body, thus preventing their reaction with DNA (Lee & Surh, 2005; Surh et al., 2005). The induction of phase II antioxidant enzymes by chemoprotective agents has been demonstrated to be an effective strategy for protecting against multistage carcinogenesis in experimental animals as well as clinical trials (Dick & Kensler, 2002; Links & Lewis, 1999).

Epidemiological studies indicate that increased consumption of cruciferous vegetables such as broccoli, Brussels sprouts, and cabbage is correlated with a decreased risk of developing cancers of the pancreas, lung, colon, and prostate (Lampe & Peterson, 2002). It is hypothesized that much of this chemoprotective effect can be attributed to the physiological effects of isothiocyanates (Bianchini & Vainio, 2004). Isothiocyanates are derived from a family of compounds known as glucosinolates, which are secondary plant metabolites that are relatively unique to cruciferous vegetables (Keum et al., 2005). Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane), a naturally occurring isothiocyanate, has received particular attention because of its anti-cancer effects (Fahey & Talalay, 1999; Pham et al., 2004). This phytochemical is a potent inducer of the phase II enzymes implicated in carcinogen detoxification (Kensler et al., 2000) and is a competitive inhibitor of CYP2E1, which is involved in the activation of carcinogenic chemicals (Henderson et al., 2000). Cancer chemoprevention by sulforaphane has been observed against 9,10-dimethy-1,2-benz[*a*]anthracene-induced mammary tumorigenesis in rats (Singletary & MacDonald, 2000), azoxymethane-induced colonic aberrant crypt foci formation in rats (Chung et al., 2000), and benzo[*a*]pyrene-induced fore stomach cancer in mice (Fahey et al., 2002).

Reactive oxygen species (ROS) and free radicals are involved in a diversity of important phenomena in the process of tumor development (Cook et al., 2004). In metazoan cells, ROS are generated by mitochondria (during respiration) and by distinct enzyme systems (Adam-Vizi, 2005). Low levels of ROS regulate cellular signaling and play an important role in normal cell proliferation, and accumulating evidence suggests that overproduction of ROS is typical of cancer cells (Nicco et al., 2005). Constant activation of transcription factors (such as NF-κB and AP-1) appears to be one functional effect of elevated ROS levels during tumor progression (Rahman, 2002). Oxidative stress can also induce DNA damage that leads to genomic instability, which may contribute to cancer progression (Phillipson et al., 2002). Recently, it was shown that ROS are produced in cells stimulated with growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Frank & Eguchi, 2003). High levels of oxidative stress are seen *in vitro* without exogenous stimulation in several human carcinoma cell lines, including malignant melanoma, colon carcinoma, pancreatic carcinoma, neuroblastoma, breast carcinoma, and ovarian carcinoma (Sung et al., 2005). Antioxidant enzymes such as catalase and Mn superoxide dismutase (Mn SOD) are downregulated in tumor cells (Li et al., 1998). It is postulated that persistent oxidative stress present in cancer cells leads to cell proliferation rather than apoptosis, which is the normal cellular response to severe oxidative stress. Thus, ROS are thought to play multiple roles in tumor initiation, progression, and maintenance.

Metallothionein (MT) is a cysteine-rich protein that can bind heavy metal ions such as copper and zinc. It is also an efficient scavenger of free radicals because of its high thiol content (Vasak, 2005). The concept is supported by the fact that the MT can scavenge hydroxyl radicals *in vitro* (Ebadi et al., 1998) and provide protection against radiation damage (Cai & Cherian, 2003). Multiple lines of evidence suggest that some chemicals that stimulate the production of ROS can also increase MT levels. Ashino et al. (2003) has also implicated MT as a regulator of heme oxygenase-1, suggesting MT as a potential target for chemoprevention by anti-cancer agents. The aim of this paper is to focus on molecular mechanisms underlying MT expression, and the significance of targeted induction of MT by sulforaphane as a strategy for achieving chemoprevention and chemoprotection.

2. Chemopreventive effect of sulforaphane

Many studies report a strong inverse relationship between the intake of cruciferous vegetables and the risk for many types of cancers. This association has been found to be stronger than the association between cancer risk and fruit and vegetable intake in general (Lampe & Peterson, 2002). It is hypothesized that much of this chemopreventive effect can be attributed to the physiological effects of glucosinolate-derived isothiocyanates (ITCs). Depending upon the structure of the specific glucosinolate and the existing reaction conditions, ITCs or nitriles usually constitute the majority of aglucons (Johnson, 2002). In broccoli, the primary glucosinolate is glucoraphanin [4-(methylsulphinyl)butyl glucosinolate], which yields sulforaphane [1-isothiocyanato-4-(methylsulphinyl)butyl isothiocyanate] and sulforaphane nitrile (SF nitrile) [5-(methylsulphinyl) pentane nitrile] as its primary aglucon products following myrosinase-dependent hydrolysis (Zhang, 2004).

Sulforaphane has been shown to be protective against carcinogen-induced tumorigenesis in various experimental models (Singletary & MacDonald, 2000; Chung et al., 2000; Fahey et al., 2002). Evidence is accumulating to indicate that sulforaphane can suppress proliferation of cancer cells in culture by causing cell cycle arrest and apoptosis induction (Jakubikova et al., 2005; Tang & Zhang, 2005; Juge et al., 2007). In human colon cancer cell line HT29, sulforaphane induces apoptotic cell death through the pro-apoptotic protein Bax, release of cytochrome *c* into the cytosol, and cleavage of poly(ADP-ribose) polymerase (PARP) (Frydoonfar et al., 2004). Administration of sulforaphane reduced cellular viability and induced apoptosis as indicated by PARP cleavage and increased release of histone associated DNA fragmentation in DU145 prostate cancer cells (Cho et al., 2005). Most interestingly, the *N*-acetylcystenine (NAC) conjugate of sulforaphane was shown recently to inhibit histone deacetylase activity (Myzak et al., 2004). Chemoprotection with sulforaphane also results in the delayed appearance of tumors.

In preclinical rodent models, there is significant data supporting the chemopreventive effects of sulforaphane at several stages of carcinogenesis. Dietary administration of sulforaphane and dibenzoylmethane alone or combination significantly inhibited the development of intestinal adenomas in the $Apc^{min/+}$ mice model (Shen et al., 2007). Moreover, sulforaphane supplementation also decreased polyp formation in $Apc^{min/+}$ mice (Hu et al., 2006). This result is corroborated by a recent finding by Myzak et al. (2006) who found that long-term treatment of sulforaphane suppressed tumorigenesis in $Apc^{min/+}$ mice through inhibition of histone deacetylase. In these experiments, a significant decrease in intestinal polyps and an increase in global acetylated histones H3 and H4 were observed, with specific increases at the *Bax* and *p21* promoters. In PC3 xenograft studies, dietary sulforaphane supplementation resulted in slower tumor growth and significant histone deacetylase (HDAC) inhibition in the xenografts, as well as in the prostate and circulating peripheral blood mononuclear cells (Myzak et al., 2007). From these studies it can be concluded that HDAC inhibition represents a novel chemoprevention mechanism by which sulforaphane can promote cell cycle arrest and apoptosis.

Sulforaphane has been shown to be a potent inducer of antioxidant response element (ARE)-regulated phase II enzymes (Thimmulappa et al., 2002; Keum et al., 2003; McWalter et al., 2004; Zhang et al., 1992). Nrf2, a member of the NF-E2 transcription factor family, induces phase II enzymes by binding to the ARE region of the promoter. Sulforaphane, as well as phenethyl isothiocyanate, differentially regulates the activation of MAP kinases and Nrf2, ARE-mediated luciferase reporter-gene activity, and phase II enzyme gene induction (Keum et al., 2003). Under basal conditions, Nrf2 is suppressed by binding to Keap1, a cytoplasmic protein anchored to the actin cytoskeleton. Sulforaphane directly interacts with Keap1 by covalent binding to thiol groups of this inhibitory protein (Hong et al., 2005). Consistent with its proposed role in Nrf2 regulation, sulforaphane is chemoprotective in wild-type animals, but loses its efficacy in the reduction of benzo[*a*]pyrene-induced gastric tumors in Nrf2 deficient mice (Ramos-Gomez et al., 2003). Analysis of gene-expression profiles by oligonucleotide microarray reveals that sulforaphane treatment results in upregulation of NQO1, GST, and GCL in the small intestine of wild-type mice, whereas Nrf2-null mice display much lower levels of these enzymes (Thimmulappa et al., 2002). Enzyme induction has also been observed in cell lines including human hepatoma (HepG2), BPrc1 p450-deficient mutant, and human adult retinal pigment epithelial cells (ARPE-19), as well as in organs (liver, stomach, small intestine, and lung) of sulforaphane fed mice (Hintze et al., 2003). A recent study in the rat reported plasma concentrations of sulforaphane on the order of 20 μM, and the most robust cluster of genes is the MT-like genes (MT-1/2 and MT-1a), which increased up to 10-fold, by 2–4 h after sulforaphane dosing (Hu et al., 2004). In mice, treated by gavage with 9 μM sulforaphane per day for 1 week, several classes of genes were identified as targets of sulforaphane in the transcriptional microarray, including cellular NADPH regenerating enzymes, xenobiotic metabolizing enzymes, antioxidant enzymes, and biosynthetic enzymes of glutathione and glucuronidation conjugation pathways (Thimmulappa et al., 2002). Furthermore, mice treated by gavage with 15 μM sulforphane per day for 5 days had an increase in quinone reductase and glutathione S-transferase activities in the liver, forestomach, glandular stomach, proximal small intestine, and lungs (Zhang et al., 1992), establishing that sulforaphane also induced phase II enzymes *in vivo* following oral administration. An increase in γ-glutamylcysteine synthetase activity was also observed with sulforaphane treatment in human prostate cells (Brooks et al., 2001). The result of these studies clearly demonstrated that responses to sulforaphane are to increase cellular defenses, leading to enhanced carcinogen detoxification and protection against potential mutagenic events.

3. Metallothioneins and chemoprevention

Every living organism must detoxify nonessential metals and carefully control the intracellular concentration of essential metals. MTs are a family of low molecular mass (6–7 kDa), cysteine-rich, inducible, intracellular proteins that bind heavy metals with high affinity (Coyle et al., 2002). The physiological functions of MTs have not yet been fully resolved. However, it is generally agreed that MTs play an important role in the homeostasis of essential metals and in the detoxification of heavy metals (You et al., 2002). In addition, MTs have been suggested to play an antioxidant role, since they contain sulfhydryl groups (Jeong et al., 2004).

There are four types of MTs, namely the MT-I, MT-II, MT-III, and MT-IV isoforms, in mammals, but the positions of cysteine are the same among these isoforms. Generally, MT is used to indicate the MT-I and MT-II isoforms. MT-I and MT-II are expressed in nearly all organs of the body, and are induced by a wide variety of stressors including heavy metals and oxidative stress-inducing agents. MT-III is an MT isoform isolated based on its inhibitory effect on neuron proliferation, and its expression is localized in the brain (Palmiter et al., 1992). The mechanism of this inhibition and the physiological significance of MT-III are not clear. MT-III regulation has been studied in a number of animal models of brain damage (Masters et al., 1994); such studies have suggested that this MT isoform is involved in reparative and/or protective processes in the brain. Moreover, it has been recently shown that mice deficient in MT-III are more susceptible to seizures induced by kainic acid and exhibit greater neuronal injury than normal mice, and that transgenic mice containing elevated levels of MT-III were more resistant to neuronal injury (Carrasco et al., 2000). These results suggest that MT-III could play a neuroprotective role; however, the mechanisms underlying such a protective role have not been fully elucidated. MT-IV was identified as a gene induced in the differentiation of stratified squamous cells and is expressed specifically in epithelial cells of organs such as the tongue and skin (Quafe et al., 1994). The function of MT-IV remains unknown. There are two forms of MT protein, the metal-bound and metal-free (apo-MT) forms. Usually apo-MT is transiently expressed in non-neoplastic cells under Zn-deficient conditions. However, in a study conducted on solid tumors from mice and rats, persistently elevated levels of apo-MT were detected in a number of tumors without any overt evidence of Zn deficiency. Although not all primary functions of the MTs have been defined with certainty, these proteins participate in different

biochemical reactions occurring *in vivo* by virtue of their high cysteine contents.

Different types of factors, such as heavy metals (Zn, Cd, Co, Ni, Ag, Hg, and Bi), glucocorticoids (e.g. dexamethasone), some alkylating agents (e.g. iodoacetate), oxidants/antioxidants [e.g., H_2O_2, *tert*-butylhydroquinone (TBHQ)], and inflammatory signals (lipopolysaccharide) can induce MT expression (Andrews, 2000). Induction by any of these compounds (except heavy metals) results in the accumulation of zinc bound to the newly synthesized apo-MT. The functions of MTs are uncertain, but they can detoxify heavy metals, provide a reserve of zinc and protect against oxidative stress (Lichtlen & Schaffner, 2001). Induction of MT genes by metals requires multiple metal response element (MRE) sequences located in the promoter region and the zinc-finger transcription factor, MTF-1 (Palmiter, 1994). Treatment of cells with metals results in translocation of MTF-1 to the nucleus and binding to MREs (Saydam et al., 2001). Direct binding of zinc to regulatory sites on MTF-1, activation of signal transduction pathways leading to covalent modification of MTF-1, and release of MTF-1 from regulatory molecules have all been proposed (Adams et al., 2002). The induction of MT-I and MT-II by glucocorticoids is mediated by the glucocorticoid receptor binding to a pair of glucocorticoid-response element (GRE) sequences upstream of the MT-II gene (Kelly et al., 1997). The MT-I gene contains an antioxidant response element (ARE) sequence similar to those found in rat phase II genes NAD(P)H:quinine oxidoreductase (NQO1) and glutathione S-transferase A1 (GSTA1). In addition to the hybrid MLTF/ARE regulating mouse MT-I, many other MT promoters contain a perfect consensus ARE (Dalton et al., 1994). The identification of MTs as stress proteins suggests that coordinated transcription of MT genes and other stress protein genes (including those encoding detoxifying enzymes) constitutes a broad range response to the presence of free radicals.

4. Modulation of metallothionein gene expression by the chemopreventive agent sulforaphane

4.1. *Transcriptional induction of the MT gene by sulforaphane*

MT expression is primarily controlled at the level of transcription. Transcription can be induced by a variety of physiological agents and environmental stressors such as transition metals, glucocorticoids, cAMP, phorbol esters, alkylating agents, oxidizing agents, ultraviolet and ionizing radiation, and phytochemicals (Jeong et al., 2004). Inducible transcription is mediated by a variety of other regulatory elements located in promoter/enhancer regions of MT genes, which include glucocorticoid-response elements, cAMP response elements, AP-1 elements, and antioxidant response elements (Dalton et al., 1994). Sulforaphane is one member of the isothiocyanate class of cancer chemopreventive compounds that has been shown to be effective in blocking initiation and progression of carcinogenesis (Fahey & Talalay, 1999; Pham et al., 2004). Several studies have demonstrated that sulforaphane can potently induce phase II detoxifying enzymes, which contributes to its chemopreventive functions (Thimmulappa et al., 2002; McWalter et al., 2004). Furthermore, sulforaphane was shown to effectively induce phase II MT gene (MT-I and MT-II) in rat livers *in vivo* by 4967 oligonucleotide microarray (Hu et al., 2004). However, the molecular signaling mechanism by MT gene upregulation is not completely understood. We have found that the levels of both MT-I and MT-II mRNA increase in a concentration-dependent manner upon treatment of cells with sulforaphane (Yeh & Yen, 2005). This result is consistent with a previous report in which sulforaphane treatment induced MT gene expression in rats (Hintze et al., 2003). Therefore, it can be inferred that sulforaphane might regulate MT mainly at the transcriptional level.

Next, we tested the ability of sulforaphane to increase MT protein expression by Western blot. Densitometric quantification of the visualized bands of MT protein revealed a significant induction of MT protein by sulforaphane in a dose- and time-dependent manner. To our knowledge, this is the first reported Western blot analysis of MT protein expression in response to *in vitro* exposure to sulforaphane (Yeh & Yen, 2005). The enhanced MT protein expression seen in HepG2 cells after treatment with sulforaphane corresponded to the induction of MT gene expression, suggesting that the observed induction of MT protein synthesis by sulforaphane is due to activation of MT gene transcription.

4.2. *Nrf2 (NF-E2-related factor 2) and MT expression*

It has been reported that MT-I and MT-II are induced in a wide range of cell types by different classes of inducers, consistent with their roles in zinc and copper homeostasis as well as defense against metal overload and oxidative stress (Palmiter, 1998). The regulation of MT gene expression is complex, involving a number of different transcription factors and signaling pathways. The ARE in the upstream promoter region of the MT gene and the transcription factor Nrf2 play key roles in the transcriptional regulation of the MT gene by multiple inducers (Dalton et al., 1994). Nrf2 is usually co-localized with Keap1 protein in the cytoplasm. After activation by an inducer, Nrf2 disassociates from Keap1 and translocates into the nucleus. Once in the nucleus, activated Nrf2 dimerizes with other cofactors, such as mafG, and binds to the AREs, activating MT gene expression (Motohashi et al., 2004). Although the transcription factors and *cis*-acting elements required for induction by antioxidants and glucocorticoids have been identified (Kelly et al., 1997), the factors and binding sites mediating induction by sulforaphane stimulation have not been established. We have shown that sulforaphane can activate Nrf2 (Yeh & Yen, 2005). The amount of Nrf2 protein expression is significantly increased by sulforaphane treatment in HepG2 cells, suggesting that increased expression of Nrf2 protein may play a key role in sulforaphane-induced MT gene activation.

4.3. *Modulation of mitogen-activated protein kinases (MAPKs) by sulforaphane*

The mitogen-activated protein kinases (MAPKs), characterized as proline-directed serine/threonine kinases, are important cellular signaling components that convert various

extracellular signals into intracellular responses through serial phosphorylation cascades (Cuschieri & Maier, 2005). At the present time, three distinct but parallel MAP kinase cascades (ERK, JNK, and p38) have been identified in mammalian cells (Kong et al., 2001a). Once activated, these three MAPKs can phosphorylate many transcription factors, ultimately leading to changes in gene expression (Kong et al., 2001b). Given the fact that MAPKs are activated by such a wide range of factors, these signaling cascades may serve as common mechanisms that integrate signaling pathways to control cellular responses to various extracellular stimuli, including xenobiotics and pharmacological agents (Owuor & Kong, 2002). MAPK pathways, in particular the ERK and/or p38 MAPK, have been reported to participate in the xenobiotic-induced activation of MT genes (Haq et al., 2005). Previous studies have also shown that overexpression or activation of MAPKs differentially affects Nrf2 activity and phase II detoxifying enzymes (Yu et al., 2001). In addition, it has been shown that induction of phase II detoxifying enzymes by phenethyl isothiocyanate (PEITC) is dependent on c-JNK activation (Yu et al., 1996; Keum et al., 2003), and the induction of NQO1 by isothiocyanate treatment is mediated by ERK pathways (Thornalley, 2002).

Recently, studies on MT induction by oxidative stress have shown that MAPK pathways are responsible for the transduction of signals to initiate MT gene activation (Haq et al., 2005). Both ERK and p38 MAPK pathways are involved in the induction of MT expression by sulforaphane in HepG2 cells (Yeh & Yen, 2005). ERK1/2 is activated by sulforaphane. Compared with untreated HepG2 cells, sulforaphane-treated cells have higher levels of p-ERK and p38 MAPK, but not p-JNK. Inhibition of the ERK1/2 MAPK pathway by PD98059 or inhibition of the p38 MAPK pathway by SB203580 partially blocks the increase of MT protein expression, and inhibition of both pathways together almost completely blocks the sulforaphane-induced MT protein expression, suggesting that both ERK and p38 MAPK are important for MT gene induction by sulforaphane. In a previous study, Shen et al. (2004) reported that activation of MAPK pathways induces ARE-mediated gene expression via an Nrf2-dependent mechanism.

5. Contribution of sulforaphane to the induction of apoptosis

Apoptosis is one of the major mechanisms of cancer suppression. It is a highly regulated process that involves activation of a series of molecular events, leading to cell death that is characterized by cellular morphological change, chromatin condensation, and apoptotic bodies which are associated with DNA cleavage (Johnstone et al., 2002). Recently, apoptosis was suggested as a novel target for cancer chemoprevention (Sun et al., 2004). Sulforaphane has been reported to induce apoptosis in various cancerous or transformed cells in culture, in chemically induced mouse skin tumors, and in transplanted tumors in nude mice by activating both extrinsic and intrinsic pathways of cell death machinery (Gills et al., 2006; Singh et al., 2004). Multiple lines of evidence suggest that sulforaphane induces apoptosis by activating pro-apoptotic signaling molecules as well as inhibiting anti-apoptotic molecules of the intracellular signal transduction pathways. Fimognari et al. (2002) reported that sulforaphane treatment leads to cell cycle arrest in G1 phase by a significant down-modulation of cyclin D3 in transformed human T lymphocytes. Moreover, sulforaphane treatment has been shown to cause growth arrest and apoptosis in the human pancreatic cancer cell lines MIA PaCa-2 and PANC-1, and the production of ROS is involved in the mechanism of sulforaphane action (Pham et al., 2004). Glioblastoma is the most malignant and prevalent brain tumor that still remains incurable. Very recent results demonstrated the ability of sulforaphane to induce apoptosis in a tumorigenic malignant U-87MG glioblastoma cell line through the activation of multiple molecular mechanisms (Karmakar et al., 2006). Most notably, 4-methylsulfinyl-3-butenyl isothiocyanate did not affect the survival of normal peripheral blood lymphocytes, suggesting that the compound induced apoptosis selectively in cancer cells (Papi et al., 2008). Our results indicate that sulforaphane has a strong growth-inhibitory effect with doses ranging from 30 to 100 μM (Yeh & Yen, 2005). The estimated IC_{50} value of sulforaphane was 65.2 μM.

In another study, sulforaphane was shown to sensitive various cells to TNF-related apoptosis-inducing ligand (TRAIL)-dependent cell death via stimulation of both receptor and downregulation of ERK and AKT apoptotic signaling pathways (Jin et al., 2007). Likewise, pretreatment of human hepatoma cells (HepG2 and Hep3B) cells with sulforaphane resulted in TRAIL-mediated cells death by multiple mechanisms involving downregulation of inhibitor of apoptotic proteins (IAPs), and subsequent activation of caspases (Kim et al., 2006). The induction of apoptosis stimulates endonuclease that cleaves DNA into oligonucleosome length fragments, resulting in a typical ladder in DNA electrophoresis, one of the hallmarks of apoptotic cell death (Montague & Cidlowski, 1996). As evidenced by DNA fragmentation, it appears that apoptosis is the main cause of cell death in the presence of sulforaphane. The apoptosis-inducing effect of sulforaphane in $HepG_2$ cells appeared in a concentration- and time-dependent manner. Similar to a previous report (Misiewicz et al., 2003), our observations suggest that treatment sulforaphane induces apoptotic cell death in human hepatoma cells.

Multiple lines of evidence indicate that apoptosis can be triggered by the activation of a set of death effector cysteine proteases called caspases with specificity for Asp-X bonds, and their activations play important roles during apoptosis. In most of apoptotic processes, caspase 3 plays a pivotal role in the terminal execution phase of apoptosis induced by diverse stimuli (Thornberry & Lazebnik, 1998). Caspases can be grouped into 'apoptotic initiator', such as caspase 8, and 'apoptotic effector', such as caspase 3, according to their substrate specificities and target proteins (Kaufmann & Hengartner, 2001). Caspase 3 is a cysteine protease that exists as an inactive zymogen in cells and becomes activated by sequential proteolytic events that cleave the 32 kDa precursor at aspartic acid residues to generate an active heterodimer comprising of 20 and 12 kDa subunits (Nicholson et al., 1995). Activation of caspase 3 leads to the cleavage of a number of proteins, including PARP. Although PARP is not essential for cell death, the cleavage of PARP is another hallmark of apoptosis. Gingras et al. (2004) found that sulforaphane-induced

medulloblastoma cell death by apoptosis, as determined by DNA fragmentation and chromatin condensation. Medulloblastoma apoptosis is correlated with the activation of caspases 3 and 9, resulting in the cleavage of PARP and vimentin. In addition, sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards the growth of PC-3 xenografts *in vivo* (Singh et al., 2004). Our results show that sulforaphane inhibits the growth of human hepatoma cells and induces apoptosis, as shown by the proteolytic degradation of PARP by caspase 3 (Yeh & Yen, 2005).

Numerous investigations have revealed the importance of ROS in apoptosis induced by various stimuli (Hildeman et al., 1999), including sulforaphane. Sulforaphane was shown to induce Chk2-mediated phosphorylation of Cdc25C in PC-3 human prostate cancer cells, resulting in G2/M phase cell cycle arrest (Singh et al., 2004). Therefore, the role of ROS as the upstream signal to induce apoptosis in sulforaphane-treated HepG2 cells was also investigated. In our study (Yeh & Yen, 2005), we show that the free radical scavengers NAC and catalase can protect cells against sulforaphane-induced DNA fragmentation during apoptosis. NAC is a free radical scavenger and glutathione precursor, which can protect cells against oxidative damage (Zafarullah et al., 2003). Catalase is an antioxidative enzyme, which converts H_2O_2 to H_2O and O_2, and protects cells from ROS-mediated damage (Cutler, 2005). These data suggest that ROS might be present in sulforaphane-induced cells.

Members of the Bcl-2 family of proteins are critical regulators of both pro-apoptotic and anti-apoptotic pathways. Pro-apoptotic Bcl-2 family proteins include Bax, Bak, and Bcl-Xs and anti-apoptotic Bcl-2 proteins include Bcl-2, Bcl-X_L, and Mcl-1. Previous studies indicated that an increase in pro-apoptotic Bcl-2 family proteins and a decrease in anti-apoptotic Bcl-2 family proteins are associated with the process of apoptosis (Gross et al., 1999). Wang et al. (2004) found that sulforaphane treatment led to downregulation of Bcl-2 and activation of caspases in prostate cancer cells. Furthermore, upregulation of Bax, downregulation of Bcl-2, and activation of caspases 3, 9, and 8 were all involved in sulforaphane-induced cell apoptosis. Bcl-2 has been shown to form a heterodimer with Bax to neutralize its pro-apoptotic effects (Rokhlin et al., 2001). Therefore, the ratio of Bax/Bcl-2 is a decisive factor in determining whether the cell will undergo apoptosis under conditions that promote cell death. In our study (Yeh & Yen, 2005), a decrease in Bcl-2 and Bcl-X_L expression was observed in sulforaphane-treated cells. Importantly, Bax expression was upregulated in sulforaphane-treated cells for up to 24 h after treatment; hence the ratio of Bax to Bcl-2 was altered in favor of apoptosis. Our results suggest that the upregulation of Bax and the downmodulation of Bcl-2 and Bcl-X_L might be another molecular mechanism through which sulforaphane-induced apoptosis in $HepG_2$ cells. Take together, the aforementioned studies show that sulforaphane is a potent pro-apoptotic agent in several different cell lines and also *in vivo*. Its activity is related to a variety of different mechanisms leading to a strong inhibition of clonal expansion of initiated cells, or their programmed cell death.

6. Summary and perspectives

The available experimental evidence suggests that it is worth testing sulforaphane as a cancer therapeutic agent. All published observations indicate that sulforaphane leads to a strong modification of cell signaling pathways including induction of Nrf2 transcription factors. In conclusion, as summarized in Fig. 1, our study demonstrated that low concentrations of sulforaphane activate the ERK and/or p38 MAPK pathways, which may lead to the induction of phase II MT gene expression though the Nrf2 transcription factor, resulting in protection and/or survival mechanism. However, exposure to high concentrations of sulforaphane might generate an oxidant signal to stimulate caspase 3 pathway activation and DNA fragmentation, leading to cell death. Free radical scavengers including *N*-acetyl-L-cysteine and catalase effectively inhibited apoptosis induced by sulforaphane. Further-

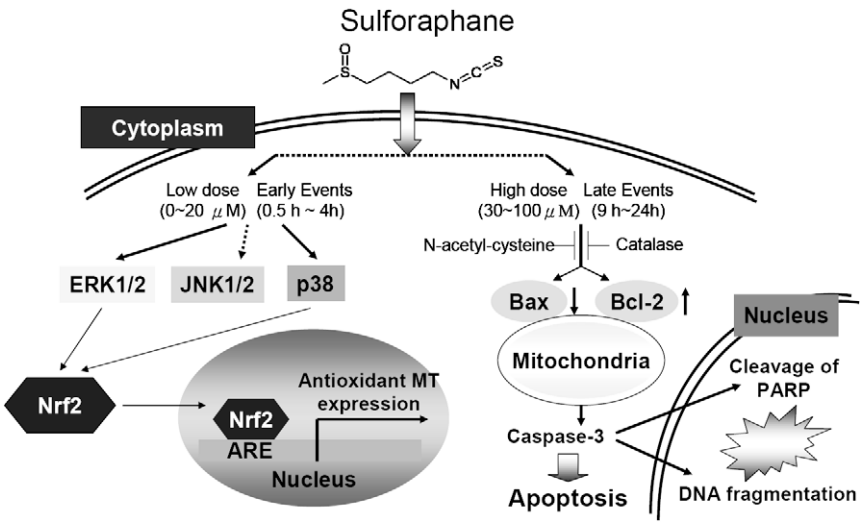

Fig. 1 – Signal transduction pathways affected by sulforaphane treatment leading to upregulate metallothionein gene (low dose) or cellular apoptotic responses (high dose).

more, these effects were found to be correlated with a shift in Bax/Bcl-2 ratio towards apoptosis. Our result is the first evidence that sulforaphane-mediated induction of a phase II detoxifying enzyme, MT, and apoptosis in human hepatoma HepG2 cells. Taken together, our study provides evidence that sulforaphane may be useful in the chemopreventive treatment of human hepatoma.

Acknowledgement

This research work was partially supported by the National Science Council, NSC 95-2313-B-005-066-MY3, Republic of China.

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