Mitochondrial Reactive Oxygen Species in Proapoptotic Effect of Promising Cancer Chemopreventive Phytochemicals

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Abstract Cancer chemopreventive phytochemicals have been identified from multiple dietary plants as well as from components of alternative medicine. Preclinical studies using rodent cancer models have provided compelling experimental evidence for cancer chemopreventive effects of these phytochemicals. Mitochondria-derived reactive oxygen species (ROS) play a critical role in their prodeath and chemopreventive responses. These phytochemicals inhibit mitochondrial electron transport chains causing ROS production, thus triggering apoptotic and/or autophagic cancer cell death. Although normal epithelial cells are resistant to mitochondrial perturbations by many phytochemicals, underlying mechanisms of the differential response in cancer cells versus normal cells remain elusive. This chapter reviews experimental evidence linking mitochondrial reactive oxygen species in cancer chemopreventive effects of a few promising phytochemicals.

Keywords Isothiocyanates • Sulforaphane • Withaferin A • ROS • OXPHOS • Apoptosis • Chemoprevention

Abbreviations

ITCs	isothiocyanates
ROS	reactive oxygen species
MRC	mitochondrial respiratory chain
PEITC	phenethyl isothiocyanate
BITC	benzyl isothiocyanate
SFN	D,L-sulforaphane
RES	resveratrol
WA	withaferin A
MOMP	mitochondrial outer membrane permeabilization
GSH	glutathione
OXPHOS	oxidative phosphorylation

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Introduction

Cancer chemoprevention, a term originally introduced by Michael Sporn, was intended to reduce the cancer burden by delaying, inhibiting, or reversing the process of carcinogenesis with the use of natural (i.e., dietary constituents) or synthetic agents. This concept has been integrated into clinical practice to some extent as exemplified by selective estrogen receptor modulators (e.g., tamoxifen) and aromatase inhibitors (e.g., exemestane) for chemoprevention of breast cancer (Fisher et al. 1998; Goss et al. 2011). A number of small molecules derived from edible vegetables or medicinal plants are also under intense research scrutiny for possible use to prevent cancer (Hecht 1999; Surh 2003; Garodia et al. 2007; Powolny et al. 2012; Singh and Singh 2012). Initial evidence for the existence of cancer chemopreventive phytochemicals in dietary plants was offered by population-based observational studies suggesting an inverse association between intake of certain fruits and vegetables and cancer risk (Verhoeven et al. 1996; Kolonel et al. 2000; Greenwald et al. 2001). Cancer chemopreventive phytochemicals with in vivo efficacy in preclinical rodent models have now been identified from several edible plants, including isothiocyanates (ITCs) from cruciferous vegetables (e.g., watercress, broccoli, mustard), allyl sulfides from garlic, resveratrol (RES) from red grapes, lupeol from mango, delphinidin from pigmented fruits, and curcumin from turmeric to name a few (Antony and Singh 2011; Powolny et al. 2012; Greenlee 2012). Demonstration of efficacy in suitable animal models coupled with a molecular understanding of the mechanisms underlying chemopreventive response is essential for clinical development of promising agents. Consistent with this notion, significant effort has been devoted to mechanistic characterization of naturally occurring cancer chemopreventive agents. A mechanistic paradigm emerging from these studies is that many of these cancer chemopreventive phytochemicals target mitochondria to cause destruction of cancer cells. This chapter is not intended to catalogue every naturally occurring phytochemical supporting this mechanistic model. Instead, the main purpose here is to exemplify a select number of agents for which the experimental evidence linking mitochondria-derived reactive oxygen species (ROS) to their proapoptotic effect is compelling.

Mitochondria are involved in diverse but interrelated physiological functions (reviewed by Nunnari and Suomalainen 2012). The impact of mitochondrial function on cellular physiology is not restricted to generation of ATP through oxidative phosphorylation as they are engaged in numerous other biochemical reactions at the intersection of multiple physiological processes including signaling, regulation of intracellular Ca^{2+} homeostasis, ROS generation, and apoptosis (Nunnari and Suomalainen 2012). Mitochondrial involvement in multiple aspects of carcinogenesis and tumor progression have also been reviewed extensively (Carew and Huang 2002; Gogvadze et al. 2008; Scatena 2012). Mitochondria are considered a valid cancer therapeutic target due to their role as integrators of prodeath and prosurvival pathways (Fulda et al. 2010; Fulda and Kroemer 2011; Wenner 2012)

Mitochondria and Cancer

Dysfunctions of mitochondria contribute to cancer initiation and progression in a complex manner (Carew and Huang 2002). Otto Warburg first described mitochondrial involvement in cancer biology (Warburg 1956). It was shown that cancer cells even in the presence of abundant oxygen exhibit increased glycolysis (Warburg effect). Impairment in the mitochondrial respiratory chain (MRC) of the tumor cells was hypothesized to be the cause of this metabolic switch. Additional differences between the mitochondria of normal versus transformed cells have been described with respect to mitochondrial membrane potential, rate of electron transfer, anion transport, protein synthesis, organelle turnover, and ROS production. Cancer cells accumulate defects in the mitochondrial genome leading to deficient mitochondrial respiration and ATP generation, ROS overproduction, and oxidative damage to mitochondria and other macromolecules (Modica-Napolitano and Singh 2004; Galluzzi et al. 2010). Germ line mutations in mitochondrial DNA have been linked to increased susceptibility to cancer development (Canter et al. 2005; Petros et al. 2005). For example, Canter et al. determined the association of the G10398A allele polymorphism in mitochondria DNA, which alters function of complex I of the MRC, with breast cancer susceptibility (Canter et al. 2005). This mitochondrial DNA polymorphism was found to be less frequent in African-American women compared with Caucasian women but associated with invasive breast cancer in African-American women with odds ratio of 2.90 (95% confidence interval 0.61−18.3; $P=0.11$) (Canter et al. 2005). In another study 11–12% of all prostate cancer patients were found to harbor mutations in complex I with alteration in conserved amino acids (Petros et al. 2005). Less than 2% of the control population exhibited mutations in complex I. Four of the conserved mutations were found in multiple independent patients with different mitochondrial DNA backgrounds (Petros et al. 2005). These authors showed further that introduction of mitochondrial DNA ATP6 T8993G mutant into the PC-3 cell line through cybrid transfer increased tumorigenic potential (Petros et al. 2005). Mitochondrial dysfunctions are linked to ROS overproduction, and impaired cell death (Carew and Huang 2002; Kroemer and Pouyssegur 2008).

Mitochondrial ROS and Cancer

The majority of cellular ROS are generated from the MRC due to a small fraction of leaky electrons that escape oxidative phosphorylation. Moderate to low levels of ROS play a role in the activation of cellular signaling pathways during host defense (Ott et al. 2007; Circu and Aw 2010; Scatena 2012). Uncontrolled ROS production can also result in oxidative damage to proteins, lipids, nucleic acid, and other biological macromolecules resulting in genotoxicity. Mutations disrupting the oxidative phosphorylation machinery result in enhanced ROS production potentially leading to a vicious cycle of increasing damage in mitochondrial DNA as well as nuclear DNA and mitochondrial dysfunctions (Canter et al. 2005; Petros et al. 2005; Scatena 2012). Furthermore, mitochondrial DNA mutations associated with ROS overproduction result in deficiency in

respiratory complex I which in turn correlates with high metastatic potential of tumor cells (Ishikawa et al. 2008). Mitochondrial ROS can oxidize the critical targets such as PKC and protein tyrosine phosphates (PTPs) in cancer cells. Also, mitogen-activated protein kinases (MAPKs) and p21 activated kinase (PAK), two classes of downstream molecules regulated by ROS, are established as the major signaling pathways for driving cancer progression. The effect of dietary antioxidants on human cancer prevention is inconsistent (Gibson et al. 2010). Cancer cells are under continuous threat of increased oxidative stress due to ROS production and can be sensitized to death by ROS-generating agents such as cisplatin, vinblastine, and dietary cancer chemopreventive agents including isothiocyanates, resveratrol, and withaferin A among others (Fulda et al. 2010; Antosiewicz et al. 2008; Low et al. 2010). This review is focused only on the anticancer phytochemicals exerting their action through ROS overproduction.

Mitochondria and Apoptosis

Mitochondria play a crucial role in regulating the controlled form of cell death (intrinsic apoptosis pathway) in response to various stimuli (Tait and Green 2010). Evasion of apoptosis is a hallmark of carcinogenic progression (Hanahan and Weinberg 2000). Mitochondrial outer membrane permeabilization (MOMP) is considered the "point of no return" for the apoptotic death cascade, triggering release into the cytoplasm of proteins that mediate cell death, such as cytochrome *c* and other apoptogenic proteins (e.g., SMAC/Diablo) (Kroemer et al. 2007; Tait and Green 2010). Bcl-2 family proteins play an important role in regulation of the MOMP (Youle and Strasser 2008). Furthermore, inner membrane permeabilization can be altered by the redox state of the mitochondrial protein vicinal thiols and through opening of the mitochondrial permeability transition pore (Ott et al. 2007). ROS can trigger apoptosis by inducing opening of the mitochondrial permeability transition pores (Circu and Aw 2010). Defects in the mitochondria-mediated intrinsic apoptosis pathway permit continued growth of neoplastic cells. The mitochondria-mediated intrinsic apoptosis pathway can be compromised by several mechanisms including: (a) overexpression or overactivation of antiapoptotic Bcl-2 family members (e.g., Bcl-2, Bcl-xL, and Mcl-1), (b) loss of expression/function of proapoptotic Bcl-2 family proteins (e.g., Bax and Bak), and (c) deregulation of proteins upstream and downstream of the Bcl-2 proteins and MOMP (Carew and Huang 2002).

Cancer Chemopreventive Phytochemicals Targeting Mitochondria

Phenethyl Isothiocyanate (PEITC)

PEITC (Fig. 1) occurs naturally as a thioglucoside conjugate in a variety of cruciferous vegetables (e.g., watercress) and released upon cutting or chewing of these

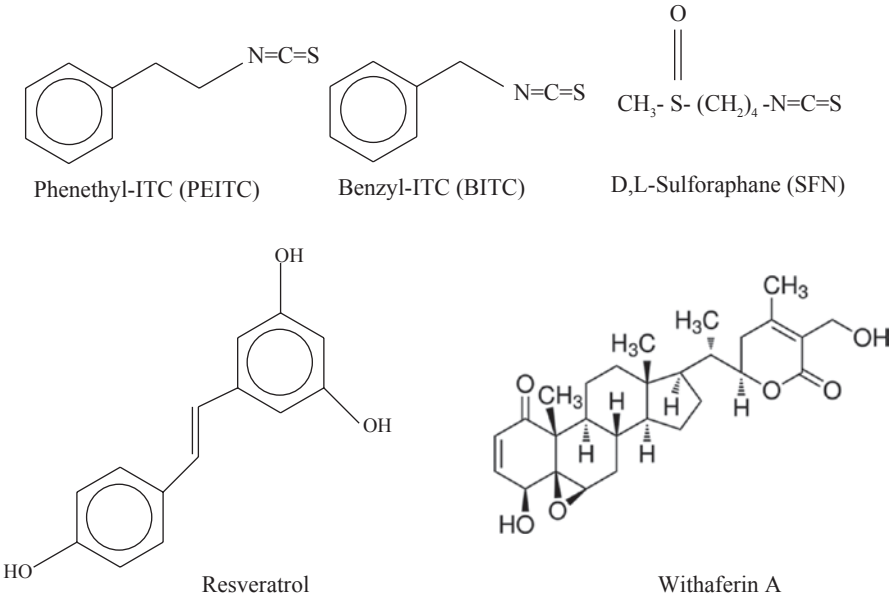

Fig. 1 Structures of the cancer chemopreventive phytochemicals reviewed in this chapter.

plants through an enzymatic hydrolytic reaction. Numerous studies, including those from our laboratory, have documented prevention of cancer by PEITC in chemically induced as well as oncogene-driven (transgenic mice) preclinical rodent cancer models (reviewed by Hecht 1995; Powolny et al. 2012; Singh and Singh 2012). Mechanistically, PEITC administration inhibits activation of carcinogens via inhibition of phase I metabolism and activation of the phase II detoxification system (Hecht 1995; Powolny et al. 2012; Singh and Singh 2012). In addition, PEITC exerts direct anticancer effects by causing apoptotic and autophagic cell death (Xiao et al. 2006; Bommareddy et al. 2009; Cheung and Kong 2010). PEITC is currently under clinical investigations in low grade B-cell lymphoma and lung cancer patients (NCT00968461, NCT00691132; http://clinicaltrials.gov).

PEITC being an electrophilic molecule readily undergoes thiocarbamoylation reaction with cellular thiols including glutathione (GSH) (Zhang 2000). PEITC-GSH conjugates can also be effluxed from the cells. Intracellular PEITC can react with cysteine thiols of cellular proteins leading to alteration in their function (Xu and Thornalley 2001; Mi et al. 2007). Several studies suggest that ROS production is an important event in proapoptotic signal transduction by PEITC in cancer cells (Rose et al. 2005; Trachootham et al. 2006; Zhang et al. 2008; Trachootham et al. 2008; Xiao et al. 2010; Xiao and Singh 2010; Powolny and Singh 2010). The mechanism of PEITC-induced ROS production and signaling downstream of ROS is fairly well characterized in prostate cancer cells (Xiao et al. 2010; Xiao and Singh 2010; Powolny and Singh 2010). Treatment of prostate cancer cells with PEITC results in suppression of oxidative phosphorylation (OXPHOS) in association with inhibition of complex III of the MRC (Xiao et al. 2010). It is intriguing

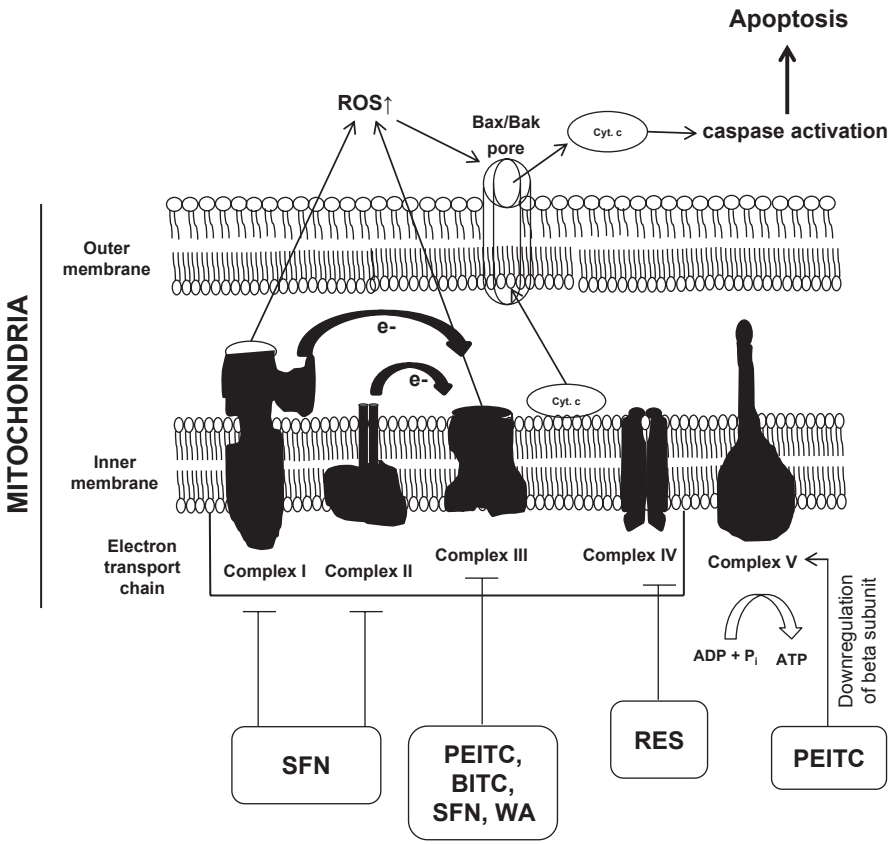

Fig. 2 A simplified cartoon depicting the role of mitochondria-derived reactive oxygen species in proapoptotic and chemopreventive response of selected phytochemicals. MRC targeted by these phytochemicals is shown.

to note that a normal human prostate epithelial cell line (PrEC) is resistant to these mitochondrial perturbations by PEITC treatment (Xiao et al. 2010). The mechanism underlying differential sensitivity of cancer cells versus normal epithelial cells to PEITC-induced ROS production is not entirely clear but PEITC treatment differentially alters expression of oxidative stress and antioxidant defense genes in PC-3 (a prostate cancer cell line) and PrEC cells (Powolny and Singh 2010). The adapter protein $p66^{Shc}$ has also been implicated in ROS production and apoptosis induction by PEITC (Xiao and Singh 2010). As summarized by a simple cartoon in Fig. 2, the events leading to PEITC-induced apoptosis downstream of ROS production involve activation of Bax, which is evident in wild-type LNCaP and PC-3 cells but not in their respective Rho-0 variants lacking OXPHOS (Xiao et al. 2010). PEITC-mediated inhibition of complex III and oxygen consumption coupled with activation of Bax have also been observed in hepatoma HepG2 cells (Rose et al. 2005). Using prostate cancer cells as a model, we have also shown previously that autophagic cell death resulting from PEITC exposure is dependent, at least in part,

on ROS production (Xiao et al. 2010). Even though the in vivo evidence for PEITC-mediated inhibition of OXPHOS or ROS production is still lacking, using a proteomics approach we have shown recently that cancer prevention by PEITC in a transgenic mouse model of breast cancer is accompanied by changes in expression of several proteins involved in cellular bioenergetics, including pyruvate kinase isozymes M1/M2 (1.33-fold decrease), mitochondrial ATP synthase H^+ transporting F1 complex beta subunit (1.38-fold decrease), hexokinase-1, isoform CRA_f (1.36-fold increase), and L-lactate dehydrogenase A chain isoform 1 (1.57-fold increase) (Singh et al. 2012). Downregulation of mitochondrial ATP synthase beta subunit protein in mammary tumors of PEITC-fed mice was confirmed by immunohistochemistry (Singh et al. 2012). However, the mechanism by which PEITC treatment inhibits complex III of the MRC is still elusive.

Benzyl Isothiocyanate (BITC)

BITC is another promising cancer chemopreventive constituent of cruciferous vegetables (e.g., garden cress) with in vivo efficacy against chemically induced and spontaneous cancer development in preclinical rodent models (Hecht 1995; Warin et al. 2009; Sehrawat and Singh 2013). BITC is structurally closely related to PEITC (Fig. 1) yet exhibits profound mechanistic differences. For example, proapoptotic protein Bim is totally dispensable for apoptosis by of BITC in MDA-MB-231 and MCF-7 breast cancer cells (Antony et al. 2012). On the other hand, Bim is critically involved in regulation of PEITC-induced apoptosis in the same cell line (Hahm and Singh 2012). At the same time, similar to PEITC, the proapoptotic response to BITC in cancer cells is critically linked to ROS production (Xiao et al. 2008; Liu et al. 2013). For example, exposure of breast cancer cells (MCF-7 and MDA-MB-231) to BITC resulted in inhibition of complex III of the MRC leading to ROS production and c-Jun NH_2-terminal kinase-dependent activation of Bax (Xiao et al. 2008). ROS production as well as apoptosis induction by BITC in breast cancer cells was significantly attenuated by overexpression of antioxidant enzymes catalase and CuZn, –SOD (Xiao et al. 2008). BITC treatment caused mitochondrial damage in rat liver epithelial RL34 cells as shown by loss of the mitochondrial membrane potential (Nakamura et al. 2002). Similar results were obtained in HL60 cells where BITC (10 μM) treatment for 3 h resulted in marked increase in the number of cells with a loss of mitochondrial membrane potential (Zhang et al. 2003). BITC-induced growth arrest and apoptosis in osteogenic sarcoma and melanoma cells was associated with ROS production (Wu et al. 2011; Huang et al. 2012). BITC treatment also inhibited growth of gefitinib resistant human non-small cell lung cancer cells via Akt/MAPK pathway and ROS generation (Liu et al. 2013). BITC-mediated inhibition of transcription factor nuclear factor-κB in lung cancer cells was shown to be ROS-dependent (Wu et al. 2010). However, this conclusion was based on protection with *N*-acetylcysteine, which is problematic because BITC, being an electrophile, can directly react with *N*-acetylcysteine limiting availability of the free reactive

agent. Similar to PEITC, the mechanism underlying BITC-mediated inhibition of complex III of the MRC is unknown.

D,L–Sulforaphane (SFN)

SFN is a synthetic racemic analogue of naturally occurring L-isomer. Talalay and coworkers were the first to demonstrate preventive activity of this agent against 9,10-dimethyl-1,2-benzanthracene-induced breast cancer in rats (Zhang et al. 1994). Subsequently, chemopreventive response to SFN was extended to other chemical carcinogens. For example, both pre- and postinitiation administration of SFN resulted in suppression of colonic aberrant crypt foci in rats induced by azoxymethane (Chung et al. 2000). We showed previously that SFN-induced apoptosis in prostate cancer cells was associated with ROS production (Singh et al. 2005). ROS production after treatment with SFN was accompanied by disruption of the mitochondrial membrane potential, cytosolic release of cytochrome *c*, and apoptosis, and all these effects were significantly blocked by overexpression of catalase. It is interesting that the SFN-induced ROS generation was significantly attenuated on pretreatment with mitochondrial respiratory chain complex I inhibitors, including diphenyleneiodonium chloride and rotenone (Singh et al. 2005). These results were somewhat unexpected as diphenyleneiodonium chloride and rotenone alone can cause ROS generation. The reasons for this discrepancy are not yet clear but could be attributable to treatment conditions. Nevertheless, unlike PEITC or BITC (Xiao et al. 2008, 2010), the ROS production by SFN was associated with inhibition of complexes I, II, and III of the MRC (Xiao et al. 2009) (Fig. 2). These results indicated mechanistic differences in ROS production between aromatic ITCs (PEITC and BITC) and thioalkyl ITC compound SFN.

Resveratrol (RES)

The cancer chemopreventive effect of RES (3,4′,5-trihydroxystilbene; Fig. 1), a polyphenol found at higher concentration in red grapes and red wine was first demonstrated by Pezzuto and coworkers (Jang et al. 1997). RES seems to affect many steps of cancer development by modulating a multitude of signaling pathways associated with cellular growth and division, apoptosis, angiogenesis, invasion, and metastasis (extensively reviewed by Pervaiz 2003; Muqbil et al. 2012).

RES-mediated biological effects could be attributed to its both pro- and antioxidant activity. A substantial amount of literature exists to support the antioxidative effect of RES in cancer (Azmi et al. 2005, 2006). For example, treatment with 50 μM RES resulted in suppression of ROS levels in PC-3 prostate cancer cells (Awad et al. 2005). On the other hand, exposure of leukemia cells to increasing concentrations of RES (0–50 μM) resulted in an increase in mitochondrial superoxide production, a decrease in transmembrane potential, and a decrease in cell viability (Low et al. 2010). Overexpression of

Bcl-2 increased mitochondrial oxygen consumption and complex IV activity, but these cells responded to the increased mitochondrial oxidative stress by RES due to a reduction in mitochondrial respiration, complex IV activity, and superoxide anion production (Low et al. 2010). In HT-29 colon cancer cells, RES caused production of superoxide anions in the mitochondria of cells undergoing apoptosis (Juan et al. 2008). Chronic treatment with RES induced redox stress and ataxia telangiectasia-mutated–dependent senescence in p53-positive cancer cells (Heiss et al. 2007). In diffuse large B-cell lymphoma cell lines, RES induced apoptosis by inhibition of constitutively activated AKT via ROS generation (Hussain et al. 2011). The RES treatment resulted in apoptosis via ROS-dependent autophagy in human colon cancer cells (Miki et al. 2012). In human acute myelogenous leukemia cells, RES synergistically potentiated vorinostat and LBH-589 lethality via ROS-mediated activation of the extrinsic apoptotic pathway (Yaseen et al. 2012). Exposure of bladder cancer cell lines to RES resulted in apoptosis in association with ROS production, decrease in ATP, cytosolic release of cytochrome *c*, and activation of caspase-9 and -3 (Lin et al. 2012).

Withaferin A (WA)

WA, a steroidal lactone isolated from the leaf and root of *Withania somnifera* (commonly known as Ashwagandha or Indian winter cherry), is another small molecule with a promising anticancer property. *Withania somnifera* is a key component of multiple Ayurvedic medicine formulations used in India for the treatment of different ailments. Evidence continues to accumulate to indicate the anticancer effect of this compound. For example, oral administration of WA for 14 weeks resulted in complete protection against 7,12-dimethylbenz[a]anthracene-induced oral carcinogenesis in hamsters (Manoharan et al. 2009). WA-mediated growth inhibition of human cancer cells implanted in athymic mice has also been reported (Srinivasan et al. 2007; Stan et al. 2008). For example, studies from our own laboratory have shown WA-mediated inhibition of MDA-MB-231 human breast cancer xenograft growth in female athymic mice (Stan et al. 2008). Similar to other phytochemicals discussed above, apoptosis induction is an important mechanism for the anticancer effect of WA (Yang et al. 2007; Stan et al. 2008; Hahm et al. 2011). A role for ROS in apoptosis induction by WA has been established in multiple cancer cell lines, including HL-60 leukemia cells (Malik et al. 2007), melanoma (Mayola et al. 2011), and breast cancer cells (Hahm et al. 2011). However, the mechanism by which WA treatment causes ROS production is well characterized only in breast cancer cells (Hahm et al. 2011). WA treatment was shown to inhibit basal and reserve OXPHOS and complex III of the MRC (Hahm et al. 2011). A normal human mammary epithelial cell line was significantly more resistant to ROS production and apoptosis induction by WA (Hahm et al. 2011). Further mitochondrial DNA-deficient Rho-0 variants of MDA-MB-231 and MCF-7 cells were resistant to WA-induced ROS production, collapse of mitochondrial membrane potential, and apoptosis compared with respective wild-type cells. In summary, WA targets complex III to trigger ROS

production leading to activation of Bax and Bak and ultimately cancer cell death (Hahm et al. 2011).

Conclusions and Future Direction

A critical review of the literature indicates that structurally divergent cancer chemopreventive phytochemicals exemplified herein possess the ability to selectively cause apoptosis in cancer cells by targeting MRC to produce ROS that serve to initiate the cell death process. Many of these phytochemicals (e.g., ITCs and WA) are electrophilic in nature, and this reactivity potentially contributes to their inhibitory effect on cancer cell MRC. However, the mechanism by which these phytochemicals inhibit MRC is still elusive as is the mechanism underlying differential sensitivity of cancer cells versus normal cells to ROS production and apoptosis induction. Finally, the in vivo validation of MRC inhibition and ROS production by these phytochemicals awaits further investigation. Emerging technologies undoubtedly will facilitate studies to fill these gaps in our knowledge. Nevertheless, it is fascinating to note that many components of our daily diet can trigger a complex set of events selectively in cancer cells leading to their elimination (death) and consequently protection against neoplasia.

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