

Dhyan Chandra *Editor*

Mitochondria as Targets for Phytochemicals in Cancer Prevention and Therapy

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 Springer

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Preface

The mitochondrion, an organelle within cells, has long been considered to be the powerhouse of the cell because of its central role in energy production. However, in the last two decades it has become clear that mitochondria also play a key role in cell survival and cell death. More recent findings further implicate a broader perspective on the role of mitochondria in multiple cellular signaling. Defects in mitochondria are associated with the genesis of multiple diseases, including cancer. One of the key functions of mitochondria is to induce cell death in multiple types of cells under physiological or environmental stresses. However, their cell-death-inducing function may be defective in cancer, causing survival and proliferation of cancer cells. Although one of the drawbacks of current cancer therapy is resistance to cell death as a result of defective mitochondrial pathways, these defects provide an opportunity to target tumor mitochondria selectively to induce cancer cell death. Selectively targeting tumor mitochondrial pathways may also decrease toxicity to normal tissues possessing normal functional mitochondria, and thus further enhance therapeutic efficacy.

During the last decade there has been significant emphasis on preventing or curing cancer with natural remedies involving the use of naturally derived phytochemicals, which possess anticancer properties with minimal toxicity. Although further studies are warranted, significant progress has been made investigating the role of mitochondria in controlling cancer cell death and proliferation in response to phytochemicals. This book describes the current status of the impact of phytochemicals on cancer cell death and survival. The key role of mitochondria in cancer prevention and therapy is also illustrated. The book contains contributions from multiple researchers working in the areas of cancer, phytochemistry, and mitochondria. This comprehensive collection of information will be useful to a broadbased audience with a focus on cancer research, prevention, and treatment.

I highly acknowledge the unwavering support and enthusiasm of all the authors and am grateful for their contributions of the chapters in the area of their expertise. I also owe a debt of gratitude to numerous researchers who reviewed the chapters and provided constructive criticism.

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OxPhos Defects and Their Role in Cancer Initiation and Progression

Nagendra Yadava, Ahmed Khalil and Sallie S. Schneider

Abstract This chapter provides a brief overview of the oxidative phosphorylation (OxPhos) carried out by five multimeric enzyme complexes. The biogenesis of the OxPhos system is very complicated because of its dual genetic origin and involvement of a large number of genes, whose products are made at two different locations, the cytosol and mitochondrial matrix. Both genetic and nongenetic factors can cause OxPhos deficiency, which can alter signaling pathways such as p53, AKT, and NF- κ B and thereby promote cancer development. A model for tumorigenesis due to OxPhos deficiency is described. This model suggests that functional decline of mitochondria with age may cause p53 suppression and thereby increase the incidence of cancer. Phytochemicals can prevent cancer development by improving OxPhos and by alleviating oxidative/redox stress and chronic inflammation.

Keywords Oxidative phosphorylation • OxPhos • Warburg hypothesis • Cancer • Tumorigenesis • Respiratory chain • p53 • Host factors • Environmental factors • Oncogenes • Tumor suppressor • Cancer metabolism

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Introduction

Oxidative phosphorylation (OxPhos) is a cellular process that uses oxygen to make ATP. Although it is the major source of ATP production, OxPhos is not essential for the survival and growth of mammalian cells until glucose becomes limiting. In the absence of OxPhos, glycolysis can meet all ATP demands of proliferative cells (Donnelly and Scheffler 1976). Therefore, the Warburg hypothesis that “injury to the respiratory chain can play a causative role in cancer development” is tenable (Warburg 1956). This is supported by the occurrence of mutations in genes involved in the OxPhos system’s biogenesis and function (Chandra and Singh 2010). In this chapter, we briefly discuss the evidence for OxPhos deficiency and its role in cancer initiation and progression. We propose that declining OxPhos could be the underlying cause of increased cancer incidence with age. Genetic and nongenetic factors may interact to increase susceptibility to tumorigenesis by exerting their effect on the OxPhos system. Phytochemicals capable of improving OxPhos, and curbing oxidative/redox stress and inflammation can prevent and halt cancer development.

OxPhos and Overall Cellular Metabolism

The Process of OxPhos

OxPhos is carried out by five multimeric enzyme complexes with the help of electron donors (NADH and protein-bound FADH_2) and electron carriers (ubiquinone and cytochrome *c*) (Fig. 1). The OxPhos complexes are: NADH-ubiquinone oxidoreductase (Complex I), succinate-ubiquinone oxidoreductase (Complex II), ubiquinol-cytochrome *c* oxidoreductase or cytochrome *Bc1* complex (Complex III), cytochrome *c* oxidase (Complex IV), and the F_0F_1 -ATP synthase (Complex V). Collectively, Complexes I to IV constitute the respiratory chain (also referred to as the electron transport chain/system). The main function of the respiratory chain is to generate a H^+ gradient across the inner mitochondrial membrane (IMM). Because this H^+ gradient drives ATP synthesis by facilitating H^+ flow, it is also referred to as the proton motive force (Δp). The Δp has two components: the mitochondrial membrane potential ($\Delta\psi_m$) and pH gradient (ΔpH ; Nicholls 2008). It drives the flow of H^+ back into the mitochondrial matrix from the inner membrane space (IMS) via Complex V, a rotary motor (Noji et al. 1997). The flow of H^+ via Complex V converts the potential energy of Δp into kinetic energy to support ATP synthesis from ADP and inorganic phosphate (Pi). Complex V makes 3ATP/rotation by using 2.7 H^+ /ATP (Wat et al. 2010). After considering the cost of Pi, ADP, and ATP equilibration across the IMM, the net cost of 1 ATP synthesis in vertebrates is suggested to be 3.7 H^+ . Complexes I, III, and IV that pump out 4, 4, and 2 H^+ , respectively, contribute toward establishing the Δp . While H^+ pumping, they simultaneously transfer electrons (e^-) liberated from the NADH and FADH_2 oxidations (Fig. 1).

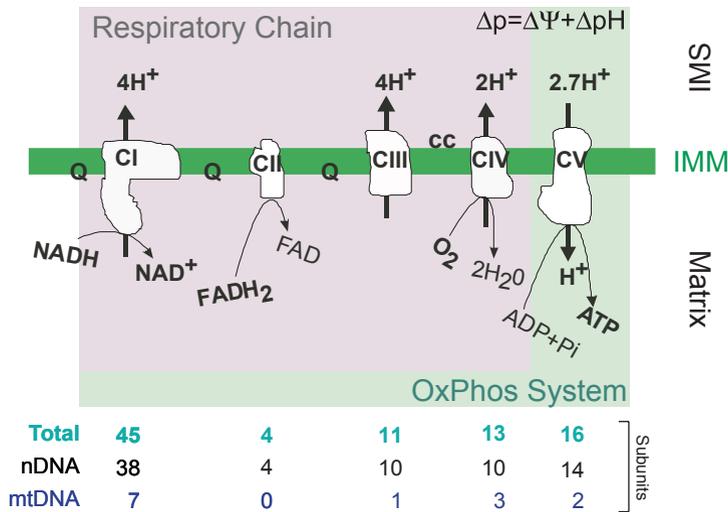
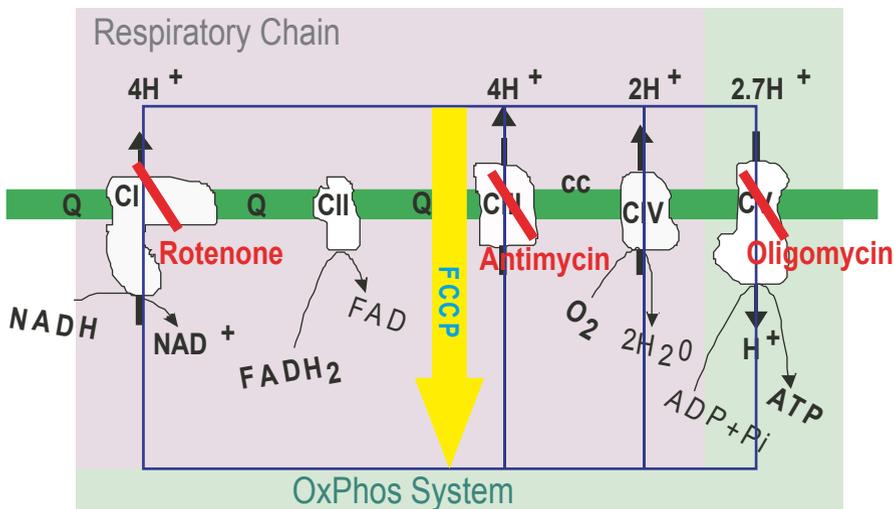


Fig. 1 The mammalian OxPhos system. Five OxPhos Complexes (CI–V) are shown with their subunit compositions, H^+ pumping stoichiometry, electron donors ($NADH$, $FADH_2$), and electron carriers (ubiquinone, Q; cytochrome *c*, CC). Respiratory chain made up by C–IV; OxPhos system: made by CI–V (or RC+CV). CI, III, IV pump out H^+ from mitochondrial matrix into the IMS (intermembrane space). Re-entry of H^+ into mitochondrial matrix drives ATP synthesis by CV. $FADH_2$ is protein bound (flavoproteins)

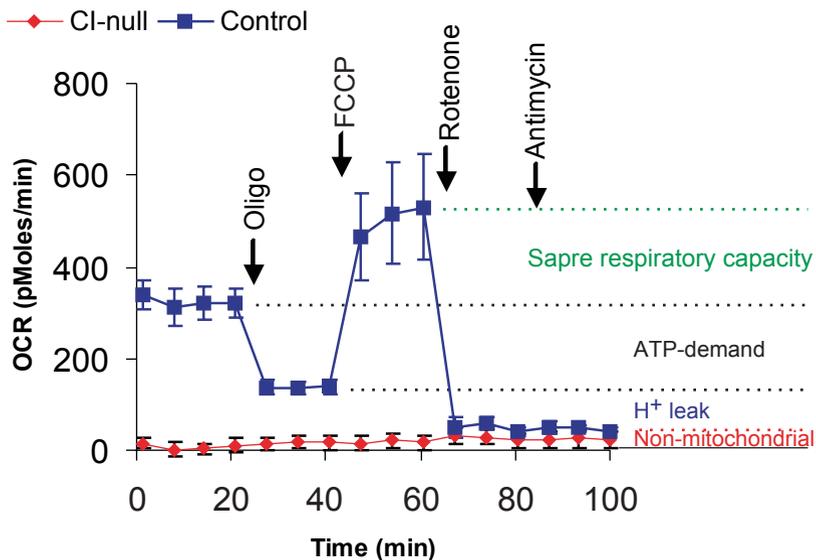
The transfer of e^- from Complexes I and II to Complex III is mediated by ubiquinone (CoQ10), and from Complex III to Complex IV by cytochrome *c*. Complex IV reduces molecular oxygen (O_2) into water by using $2e^-$ it has received from cytochrome *c*. The rate of oxygen consumption (i.e., respiration) is a good measure of the respiratory chain function.

Functional Assessment of OxPhos Using Respirometry

Respirometry using intact cells is often used to derive important information regarding OxPhos under physiological conditions. Fig. 2 shows a typical respirometry assay using intact cells. Mitochondrial and nonmitochondrial respiration can be easily determined using inhibitors such as rotenone and antimycin A (Fig. 2a). Because the IMM is not completely impermeable to H^+ , they can re-enter into the matrix without passing through Complex V. Therefore, the respiratory activity driven by the H^+ leak is not coupled to ATP synthesis. It is insensitive to oligomycin, a Complex V inhibitor (Fig. 2b). Thus, the oligomycin sensitivity test can give estimates of the respiratory activities supporting ATP-turnover and the H^+ leak (Jekabsons and Nicholls 2004). In our experience, almost every cell type has some spare (reserve) respiratory capacity, which can be determined by using an uncoupler such as the carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP, Fig. 2b).



a



b

Fig. 2 *In situ* respirometry assay using intact cells. (a) Selected cell permeable drugs commonly used to interrogate the OxPhos system by manipulating the H⁺ circuit. Respectively, rotenone, antimycin A, and oligomycin act on CI, III, and V. (b) Side-by-side comparison of the respiratory activities of normal (Control) and Complex I-deficient (CI-null) Chinese hamster lung fibroblasts. Respiration rates were measured using a 24-well extracellular flux analyzer from Seahorse Bioscience (Billerica, MA) in the presence of 15-mM glucose as the substrate. 2- μ g/ml oligomycin (Oligo), 3- μ M FCCP, 2- μ M rotenone, and 1- μ M antimycin A were added sequentially

Respiratory capacity may vary based on the carbon substrates used (e.g., glucose, pyruvate, glutamine) and the presence of oligomycin.

Because the plasma membrane is impermeable to ADP, it is not possible to determine the OxPhos capacity (the maximal ADP-stimulated respiration) using intact cells (Brand and Nicholls 2011; Clerc and Polster 2012; Dranka et al. 2011). Serial measurements with intact and permeabilized cells under the same conditions would be required. Selective permeabilization of the plasma membrane can overcome limitations in the substrate and ADP supply to mitochondria. Such serial measurements can give estimates of the OxPhos and respiratory capacities and permit assessment of the direct versus indirect effects of drugs on the OxPhos system (Brand and Nicholls 2011; Clerc and Polster 2012; Dranka et al. 2011).

Integration of the OxPhos System with Cellular Metabolism

OxPhos is dependent upon an adequate supply of NADH, FADH₂, O₂, and ADP. The major source of NADH and FADH₂ is the tricarboxylic acid (TCA, also known as citric acid or Krebs) cycle. The TCA cycle is functionally linked with glycolysis via pyruvate and NADH shuttles (Fig. 3). The NADH redox shuttles help regenerate cytosolic NAD⁺ required as cofactor for glyceraldehyde-3-phosphate dehydrogenase. Limitations in NAD⁺ supply can halt glycolysis at this step. Therefore, in the presence of limited lactate dehydrogenase (LDH) activity, the operation of NADH shuttles becomes essential to sustain glycolysis. As NAD⁺ dependent pyruvate (PDH), isocitrate (IDH3), and α ketoglutarate (KGDH) dehydrogenases are feedback-inhibited by NADH, a reduced OxPhos activity can suppress the TCA cycle. This is exemplified by CO₂ and asparagine auxotrophy observed in respiration-deficient cells (DeFrancesco et al. 1975, 1976; Ditta et al. 1976). Due to the halted TCA cycle, a limitation in oxaloacetate is thought to be the cause of asparagine auxotrophy by limiting aspartate production via transmutation that serves as a precursor for the asparagine synthesis. Therefore, tumors with OxPhos deficiency may become overdependent on asparagine for their growth. Alternatively, they may upregulate oxaloacetate synthesis via pyruvate carboxylase (PC), a key enzyme involved in the TCA cycle anaplerosis (filling-in). Thus, an OxPhos insufficiency may alter the overall balance of cellular metabolism by promoting anaplerosis and citrate exit, which may be used for lipid synthesis.

The TCA cycle anaplerosis via glutamine oxidation plays an important role in tumorigenesis. Glutamine enters the TCA cycle after its conversion into α -ketoglutarate (Fig. 3). If the OxPhos system is severely impaired, then further oxidation of α -ketoglutarate is not possible. Instead, in the presence of respiratory chain inhibitors or hypoxic conditions, glutamine undergoes reductive metabolism (Metallo et al. 2012; Mullen et al. 2012). Tumor cells use reductive glutamine carboxylation to make citrate for lipid synthesis (Mullen et al. 2012). Some tumors are addicted to glutamine, even in the presence of oxygen. Glutamine oxidation supplements the TCA cycle with oxaloacetate for citrate synthesis. The critical role of oxaloacetate is provided by overexpression of the pyruvate carboxylase (PC), which eliminates the glutamine-dependence of tumor cells (Cheng et al. 2011b).

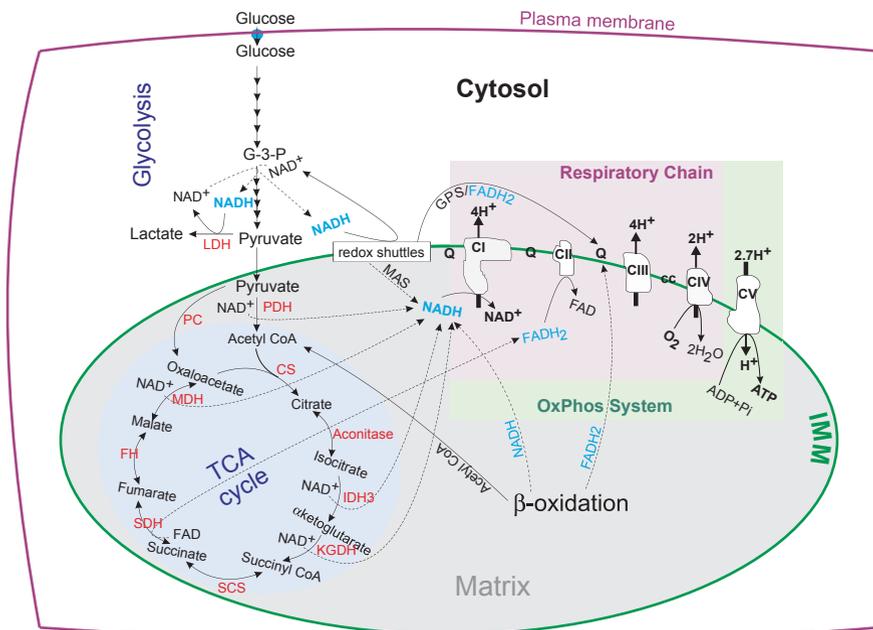


Fig. 3 Integration of the OxPhos system with overall cellular metabolism (modified from Yadava et al., 2013, Fig. 1). Glycolysis, the TCA cycle, and the OxPhos system are shown. Redox shuttles MAS: malate–aspartate redox shuttle (feeds e^- to CI); GPS: glycerol-3-phosphate redox shuttle (feeds e^- to CIII via Q). Selected enzymes lactate dehydrogenase (LDH), pyruvate dehydrogenase (PDH), pyruvate carboxylase (PC), citrate synthase (CS), aconitase, isocitrate dehydrogenase (IDH3: NAD^+ -dependent), α -ketoglutarate dehydrogenase (KGDH), succinyl-CoA synthase (SCS: ATP, GTP-dependent), succinate dehydrogenase (SDH or Complex II), fumarate hydratase or fumarase (FH), and malate dehydrogenase (MDH) are shown. The NADH and FADH₂ generation from specific reactions are shown by dashed lines. The α -ketoglutarate conversion into isocitrate by reverse reactions of the cytosolic and mitochondrial IDH1 and IDH2, respectively, supports reductive glutamine metabolism. IDH1 and IDH2 are $NADP^+$ -dependent enzymes

Citrate provides acetyl-CoA in cytosol for de novo fatty acid synthesis. This is confirmed by the suppression of tumor growth by ATP-citrate lyase inhibition (Hatzivassiliou et al. 2005). However, it is not yet clear what determines the export of citrate from mitochondria. Is it the demand for citrate in the cytoplasm, or an attenuation of the downstream reactions of the TCA cycle (e.g., IDH3)? Citrate export from mitochondria is expected to reduce net yields of NADH and ATP per pyruvate oxidation. Therefore, the balance between citrate export and its further oxidation to support OxPhos may be altered in the proliferating cells toward favoring lipid synthesis at the expense of OxPhos.

OxPhos is not essential for cell survival. In the presence of asparagine and glucose in a buffered growth medium, even complete OxPhos deficiency does not impair growth and proliferation of lung fibroblasts (Donnelly and Scheffler 1976; Yadava et al. 2002). This clearly suggests that glycolysis is capable of meeting all

ATP demands of proliferating cells. In normal lung fibroblasts only ~40% of ATP is derived from OxPhos (Compton et al. 2011; Donnelly and Scheffler 1976). This level of ATP deficiency is not detrimental unless cells are deprived of glucose, a condition that will rarely occur in vivo.

Complexity of the Functional Synthesis of the OxPhos System

The biogenesis of the OxPhos system, which has bigenomic origin, is a complicated process. It is made up of nuclear-(n)DNA and mitochondrial-(mt)DNA-encoded proteins (Fig. 1). At least 45 different proteins are required for production of Complex I, 4 for Complex II, 11 for Complex III, 13 for Complex IV, and 16 for Complex V (Ghezzi and Zeviani 2012). All complexes except Complex II have at least one mtDNA-encoded subunit. The human mtDNA is a ~16.7-kb circular molecule. It encodes 13 proteins: ND1-6 and ND4 L (Complex I subunits); Cyt b (Complex III subunit); COI-III (Complex IV subunits); and ATP 6, 8 (Complex V subunits). These proteins are made inside mitochondria using protein synthesis machinery similar to prokaryotes (Christian and Spremulli 2012). This mitochondrial protein synthesis machinery also has dual genetic origin. It involves 22 tRNAs and 2 rRNAs encoded by the mtDNA. All the ribosomal proteins and translation factors are encoded by the nDNA. In addition to structural proteins, the biogenesis of Complexes I, II, III, IV, and V also requires over 10, 2, 4, 11, and 3 assembly factors, respectively (Ghezzi and Zeviani 2012). Another layer of complexity is added by supercomplex formation by the physical association of OxPhos complexes. Supercomplexes of varying sizes formed by the associations of Complexes I, III, and IV are commonly observed. One of the benefits of supercomplex formation is its stabilizing effect on individual complexes (Acin-Perez et al. 2008; Schagger 2001). The dynamics of supercomplexes also appears to be regulated by additional proteins such as HIG2A (Chen et al. 2012b). Taken together, the assembly of a functional OxPhos system requires a large number of genes present in nuclear (nDNA) and mitochondrial (mtDNA) genomes, their coordinated expression, protein translocation across compartments, proper assembly of proteins into individual and supercomplexes, and a quality control by turning over the failed assemblies and dysfunctional complexes. This level of complexity is expected to increase the chance of spontaneous impairments of the OxPhos system.

Evidence of OxPhos Defects in Cancer

Functional Evidence for the Warburg Hypothesis

A partial irreversible injury to OxPhos in cancer development was suggested by Otto Warburg (Warburg 1956). This was based on relatively more lactate production

per unit of glucose by cancer cells than normal tissue, even in the presence of adequate oxygen, a phenomenon known as aerobic glycolysis (the *Warburg effect*). Warburg predicted that a partial respiratory injury could cause a metabolic switch toward glycolysis to meet ATP demands of cancer cells. Glycolysis provides only two net ATP molecules per glucose consumed by substrate level phosphorylation. Thus, the ATP yield from glycolysis is over fifteenfold lower than the total yield obtained from complete oxidation of one glucose molecule via OxPhos. Despite this, glycolysis alone is sufficient to support proliferation of severely respiration-deficient cells (Donnelly and Scheffler 1976). The yield of ATP from glycolysis is several-fold lower compared to OxPhos, therefore even a minor OxPhos deficiency is expected to upregulate glycolysis to meet cellular ATP demands. Although an upregulation of glycolysis is supported by increased ^{18}F -2-deoxyglucose (FDG) uptake by solid tumors, the issue of OxPhos deficiency is not yet settled (Kelloff et al. 2005; Koppenol et al. 2011; Weber et al. 2000). Functional studies of OxPhos in tumors are not yet possible. Indirect cryogenic NADH/FAD (protein bound) fluorescence imaging suggests that OxPhos may be impaired or suppressed in cancers (Xu et al. 2010, 2013). NADH and FAD are the fluorescent forms of NADH/NAD⁺ and FADH₂/FAD redox couples. An increase in NADH fluorescence would most likely be the result of impaired NADH oxidation rather than increased NADH production because of its feedback inhibition. However, an increase in FAD fluorescence could be indicative of both enhanced FADH₂ oxidation and decreased reduction of the FAD to FADH₂. In tumors, relative to the adjacent normal tissues, the NADH and FAD levels are found increased up to ~three- and fivefold, respectively (Xu et al. 2013). These changes are suggested to be due to limited oxygen supply, however, they may also arise due to OxPhos defects. Technical advances in the future may permit better assessment of OxPhos *in vivo* within tumors and their nearby environments.

Genetic Evidence for OxPhos Deficiency in Cancer

OxPhos deficiency in cancer is supported by genetic evidence. Reduced mtDNA copy number and somatic mtDNA mutations are found in breast, colon, renal, head and neck, ovarian, prostate, thyroid, and brain tumors/cancers (Brandon et al. 2006; Choi et al. 2011; Copeland et al. 2002; Modica-Napolitano et al. 2007; Tseng et al. 2011). The nucleotide polymorphisms in mtDNA are suggested to modify cancer risk (Darvishi et al. 2007; Lam et al. 2012; Theodoratou et al. 2010). Although the functional consequences of the majority of mtDNA mutations/polymorphisms are unclear, some of them can severely impair OxPhos (Gasparre et al. 2007; Horton et al. 1996; Mayr et al. 2008). Severe Complex I deficiency is commonly associated with renal and thyroid oncocytomas (Zimmermann et al. 2009, 2011). The mtDNA mutations may be selected during tumorigenesis (Gasparre et al. 2008; Gasparre et al. 2009). Mutations and altered expressions of genes such as TFAM, POLG, and SUV3 involved in mtDNA replication,

expression, and regulation are also found in cancers (Chen et al. 2012a; Guo et al. 2011; Singh et al. 2009).

Genetic defects of OxPhos are not limited to mtDNA expression/function. The catalogue of somatic mutations in cancer (COSMIC) database (<http://www.sanger.ac.uk/genetics/CGP/cosmic>) suggests that nDNA-encoded subunits and assembly factors of different OxPhos complexes are also mutated. Most of the Complex I subunits (NDUFA1-13, NDUFAB1, NDUFB1-11, NDUFC1-2, NDUFS1-8, and NDUFV1-3) and several assembly factors (NDUFAF1-4) have somatic mutations in different cancers. The mutations are mostly missense mutations. They may affect Complex I assembly, function, or stability. For example, the G32R mutation found in the NDUFA1 subunit is known to impair Complex I assembly (Potluri et al. 2009). Interactions of these mutations with the mtDNA-encoded subunits (ND1-6, 4 L) and their mutations and polymorphisms may determine their actual phenotypic severity. One clear example of such interaction is the NDUFA1 (also known as MWFE) subunit. Its stability and incorporation into Complex I is compromised based on the mtDNA background (Yadava et al. 2002, 2004). Germ-line mutations of Complex II predispose to paraganglioma, pheochromocytoma, and a few other tumors (Bayley et al. 2010; Burnichon et al. 2010; Kunst et al. 2011; Wagner et al. 2012). Mutations in all Complex II subunits (*SDHA*, *SDHB*, *SDHC*, *SDHD*) and its assembly factors (e.g., *SDHAF2*) are linked with predisposition to sporadic and familial paraganglioma (Bardella et al. 2011; Ghezzi and Zeviani 2012; Wallace 2012). Genetic polymorphisms of Complex II also modify the risk of breast and thyroid cancers in Cowden and Cowden-like syndromes (Ni et al. 2012). Germ-line mutations of Complex II predisposing to cancer provide the strongest support for the Warburg hypothesis, and suggest that impairments of OxPhos could indeed play a causative role in cancer development. Somatic mutations in cancers are not limited to Complexes I and II only. Other complexes are also affected (not shown, COSMIC database). It appears that the mutations in genes encoding the OxPhos system are widespread, however, their functional consequences and their roles in tumorigenesis remain to be determined.

Apart from the mutations, alterations in gene expression also affect the OxPhos system in cancers. Reduced expression of the β -subunit of Complex V is found in majority of cancers. Its relative expression provides a high-value prognostic tool for cancers (Cuezva et al. 2002; Isidoro et al. 2005). Increased expression of Complex V inhibitory factor 1 (IF1) and uncoupling proteins (e.g., UCP2) are also noted in tumors (Ayyasamy et al. 2011; Sanchez-Cenizo et al. 2010). Such changes can reduce OxPhos efficiency.

Role of OxPhos Defects in Cancer Initiation and Progression

Molecular Adaptations Occurring During Tumorigenesis Influence OxPhos

Tumorigenesis is a multistep process involving initiation, promotion, and progression. Genetic and epigenetic changes that impair the function of genes that suppress tumorigenesis (e.g., p53, p16, pRB, PTEN) can initiate tumorigenesis by conferring immortality to cells. Similar events can also upregulate the function of oncogenes (Ras, Myc, AKT1) directly or indirectly (Hanahan and Weinberg 2011). Constitutive activation of oncogenes confer uncontrolled growth potential to immortalized cells (transformation), which is a critical event in tumorigenesis. It equips cells with adaptive capabilities that respond to (i) limited nutrient supply, (ii) increased requirement of macromolecules (proteins, lipids, and nucleotides) biosynthesis to support growth, and (iii) hypoxia due to limited blood supply (Cantor and Sabatini 2012). Metabolic switching to support anabolic processes and reduce dependence on the availability of oxygen is one of the primary physiological responses of tumor cells to cope with hypoxic conditions. The hypoxia inducible factor 1α (HIF1 α), is a key player in facilitating adaptations to hypoxia (Cantor and Sabatini 2012; Tormos and Chandel 2010). Proteins such as Sirt3, CHCHD4, and NDUFA4L2 that localize to mitochondria directly link OxPhos activity with hypoxic response and cancer development (Finley et al. 2011; Haigis et al. 2012; Tello et al. 2011; Yang et al. 2012). Tumor cells also modify their local environment to promote angiogenesis (new blood vessel formation) and spread in the neighboring tissue (invasion) by secreting cytokines (e.g., VEGF, IL-6) and proteases (e.g., metalloproteases). Increased invasiveness also facilitates the escape of tumor cells to other locations (metastasis). The tumor suppressors and oncogenes often regulate glycolysis and oxidative metabolism in opposing directions (Levine and Puzio-Kuter 2010). Whereas the tumor suppressors promote OxPhos, the oncogenes suppress it. Therefore, their deregulation often results in metabolic reprogramming of the cells. Because cellular transformation suppresses oxidative metabolism (Yang et al. 2010), there is a clear possibility that compromised OxPhos could predispose to tumorigenesis (Gaglio et al. 2011; Kulawiec et al. 2008). Furthermore, the observations of mtDNA and p53 mutations together in cancers also support a causal link between OxPhos deficiency and cancer development (Darvishi et al. 2007; Gochhait et al. 2008; Lahiry et al. 2009).

OxPhos Deficiency Promotes Cancer Development and Predisposes to Tumorigenesis

OxPhos defects enable cells to acquire several hallmark capabilities (Yadava et al. 2013). These capabilities are: (i) sustained proliferative signaling, (ii) evasion of growth suppression, (iii) resistance to death, (iv) replicative immortality, (v) increased angiogenesis, (vi) invasiveness and metastasis, (vii) reprogrammed energy metabolism, and (viii) evasion of immune destruction (Hanahan and Weinberg 2000, 2011). OxPhos deficiency can inhibit tumor suppressors, for example, p53 and PTEN (Compton et al. 2011; Pelicano et al. 2006), activate oncogenes, for example, of AKT1 and HIF1 α pathways (Briere et al. 2005; Pelicano et al. 2006; Porcelli et al. 2009; Sun et al. 2009), induce extracellular matrix remodeling related genes (van et al. 2006), confer resistance to apoptosis (Compton et al. 2011; Dey and Moraes 2000), switch metabolism toward glycolysis (Acebo et al. 2009; Sharma et al. 2011), and cause inflammation (Kamp et al. 2011). Therefore, it can play both primary and secondary roles in cancer development. Consistent with this view, it is found that improvements in OxPhos suppress cancer progression (Santidrian et al. 2013; Wang and Moraes 2011).

The secondary role of OxPhos deficiency in cancer development is indicated by numerous studies. These studies have relied on manipulating the OxPhos system in immortalized cells and subsequently testing their transformation, tumorigenicity, and metastasis. MtDNA mutations and depletion were found to enhance transformation efficiency, tumorigenicity, and metastatic properties (Imanishi et al. 2011; Kulawiec et al. 2008; Petros et al. 2005). Partial OxPhos deficiency causes relatively more aggressive tumors compared to severe OxPhos deficiency (Park et al. 2009). Recent evidence suggests that OxPhos deficiency may also play a primary role in tumorigenesis. One study showed that maternal transmission of mtDNA mutations, originated due to the haploinsufficiency of the mitochondrial helicase mSuv3, predisposed mice to tumorigenesis (Chen et al. 2012a). Another study found that Complex I deficiency, due to a mutation in the ND6 subunit, also predisposed mice to tumorigenesis (Hashizume et al. 2012a). Although the spectrum of tumors in these mice was not similar, these studies clearly enforce the significant role OxPhos deficiency can play in predisposition to cancer.

Effects of OxPhos Deficiency

There are multiple effects of OxPhos deficiency. OxPhos defects can cause bioenergetic deficit, oxidative/redox stress, and acidosis. They can also impair the TCA cycle, alter cell fate, impair signaling pathways, and induce epigenetic changes and inflammation at the cellular and organism levels. Because many of these effects are interdependent, it is difficult to dissect out the exact role of a single factor in cancer development. Altered signaling pathways and chronic inflammation due to oxidative/redox stress could be the key players in tumorigenesis (Fatemie et al. 2012; Goh et al. 2011; Pelicano et al. 2006; Santidrian et al. 2013; Sharma et al. 2011).

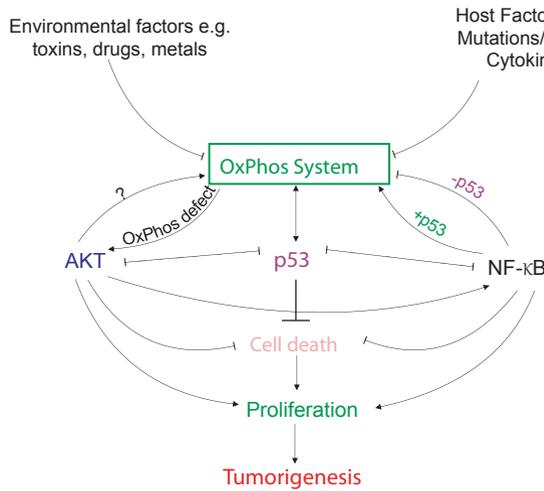


Fig. 4 A model for tumorigenesis by OxPhos deficiency. The OxPhos system's impairments suppress p53 and activate AKT. These two pathways can upregulate NF- κ B activity. Further suppression of p53 by OxPhos deficiency can negatively affect the OxPhos system. Environmental and host factors may alter the activities of these pathways implicated in tumorigenesis by impairing the OxPhos system. This model is based on the published literature and our unpublished results obtained from normal Complex I deficient cells

Effects of OxPhos Deficiency on Selected Signaling Pathways Relevant to Cancer Development

OxPhos deficiency can influence the major signaling pathways implicated in cancer development. Our working model of the tumorigenesis by OxPhos deficiency is shown Fig. 4. This is based on the regulatory relationships of the signaling pathways such as AKT, p53, and NF- κ B. They are regulated by oxidative/redox stresses, the key components of retrograde signaling from mitochondria to the cytoplasm/nucleus. OxPhos deficiency often results in oxidative/redox stress.

Although p53 acts as an intracellular stress sensor, NF- κ B is considered an extracellular stress sensor (Gudkov et al. 2011). Both regulate glycolysis and oxidative phosphorylation. p53 represses glycolysis and promotes OxPhos by regulating the expressions of the genes such as GLUT1 and 4, TIGAR, PGM, PDK2, SCO2, POLG, and so on (Liang et al. 2013; Maddocks and Vousden 2011). Depending upon the p53 status, NF- κ B can promote (in p53^{+/+} cells) or suppress (in p53^{-/-} cells) OxPhos (Tornatore et al. 2012). It positively regulates OxPhos via p53-mediated upregulation of SCO2, a Complex IV assembly factor (Mauro et al. 2011). The presence of p53 also prevents mitochondrial localization of RelA, a core component of the canonical NF- κ B pathway. Mitochondrial RelA reduces OxPhos by impairing mtDNA transcription (Johnson et al. 2011). There appears to be a reciprocal positive regulatory relationship between NF- κ B and glycolysis depending upon the

p53 function (Kawauchi et al. 2008; Salminen and Kaarniranta 2010). The NF- κ B may act as a tumor suppressor in the presence of p53, but in the absence of p53 it could become oncogenic. The switch from oxidative metabolism toward glycolysis could be a turning point in the role reversal of NF- κ B (Tornatore et al. 2012).

The p53 and NF- κ B pathways are also regulated by AKT found constitutively activated in the majority of cancers. AKT positively regulates NF- κ B and negatively regulates p53 (Das et al. 2005). AKT is the key downstream effector of the PI3K-signaling pathway, a master regulator of cell survival and proliferation (Cantor and Sabatini 2012). Therefore, the deregulation of AKT by OxPhos deficiency could become conducive for tumorigenesis (Testa and Tschlis 2005). OxPhos deficiency is consistently linked with constitutive activation of AKT via oxidative/redox stress (Pelicano et al. 2006; Sharma et al. 2011). This activation is reversible.

Complex I deficiency also reversibly suppresses p53 (Compton et al. 2011). The effects are not limited to Complex I deficiency. Impaired mitochondrial protein synthesis and Complex II deficiency also suppress p53 (P. Patel, N. Yadava, unpublished; Compton et al. 2011). Genetic variations in Complex II subunits alter p53 turnover via affecting the function of Nqo1, a FAD-dependent nonmitochondrial NAD(P)H-small ubiquinone oxidoreductase (Ni et al. 2012). Small ubiquinones do not enter mitochondria efficiently (Erb et al. 2012). Therefore, Nqo1 function is mostly restricted to the cytosol. Altered redox states of the NAD(P)H/NAD(P)⁺ and FADH₂/FAD couples play a key role in p53 regulation via Nqo1. Because p53 suppresses NF- κ B expression, a loss of p53 function can also result in NF- κ B upregulation. AKT induced by OxPhos deficiency may also upregulate NF- κ B. Therefore, it is possible that even modest OxPhos deficiency could promote tumorigenesis by suppressing p53 and activating AKT and NF- κ B pathways as shown in Fig. 4, which remains to be confirmed *in vivo*.

Role of p53 and OxPhos Interaction in Age-Dependent Cancer Incidence

The p53 protein is a major tumor suppressor that exerts its influence on different processes either as a transcription factor or by direct action. Its function is impaired in the majority of cancers either by inactivating mutations or misregulation (Brady and Attardi 2010). The tumor suppressor activity of p53 is suggested to be distinct from its activity in response to acute DNA damage (Brady et al. 2011). The critical role of p53 in preventing tumorigenesis is proven by numerous studies using stable and conditional p53 knockout mice (Christophorou et al. 2006; Hinkal et al. 2009; Kemp et al. 1994). Restoration of p53 function in tumors causes their regression (Ventura et al. 2007). In humans, p53 germ-line mutations result in Li-Fraumeni syndrome characterized by a strong predisposition to a spectrum of cancers. Breast cancer is the most common cancer in women with Li-Fraumeni syndrome (Gonzalez et al. 2009). Studies with mice suggest that the p53 function declines with age (Feng et al. 2007). Although it remains to be clearly proven in humans,

this may be one of the possible causes for the increased incidence of cancer with age. Because mitochondrial function also declines with age, the observations that OxPhos deficiency can suppress p53 are very interesting. The OxPhos deficiency-mediated p53 suppression could be the cause of increased cancer incidence with age (Yadava et al. 2013). The age-dependent decline of OxPhos due to mtDNA mutations and other factors has been suggested to cause cancer and chronic diseases (Michikawa et al. 1999; Wallace 2005, 2012). In tissues such as muscle, OxPhos clearly declines with age (Picard et al. 2010). Whether such an age-dependent decline occurs in mitotic tissues such as epithelial and stromal tissues remains to be determined. Irrespective of the cause, a decline of OxPhos could play a key role in the onset of tumorigenesis with age by suppressing p53 function while also affecting other signaling pathways (Fig. 4; Compton et al. 2011). Recent studies with mtDNA mutant mice suggest a causative role of the OxPhos deficiency, however, the role of p53 in this process remains to be explored (Chen et al. 2012a; Hashizume et al. 2012b; Mito et al. 2013).

Host and Environmental Factors Affecting OxPhos

Lifestyles, Environmental Factors, Cancer, and OxPhos

The majority of cancers arise due to exposures to host and environmental factors (Lichtenstein et al. 2000). The mitochondrial metabolism can interact with these factors and thereby increase susceptibility to tumorigenesis. These factors can modify the effects of genetic variations, stochastic events, and epigenetic changes affecting OxPhos. It is interesting to note that despite a relatively high frequency of pathogenic mtDNA mutations (1/200) in the general population, clinical mitochondrial disease is less frequent (~1/20,000; Chinnery et al. 2012). This suggests that OxPhos deficiency may be relatively common in the general population. Whether individuals with mtDNA mutations who do not develop mitochondrial disease are predisposed to cancer development remains to be explored. Given the high lifetime risk of cancer in men (~1/2) and women (~1/3) (American Cancer Society statistics 2007–2009), it is possible that the OxPhos system could interact with lifestyle and environmental factors. These factors could also modify the clinical presentation of diseases with clear mitochondrial etiology. For example, the onset of Leber hereditary optic neuropathy is influenced by alcohol intake and smoking (Kirkman et al. 2009).

Cytokines Effect on OxPhos

The presence of cytokines in the local environment could play an important role in regulating cell metabolism because inflammatory and growth-promoting cytokines

suppress oxidative metabolism. For example, tumor necrosis factor α (TNF α) impairs OxPhos in myocytes (Mariappan et al. 2012; Remels et al. 2010), adipocytes (Chen et al. 2010), hepatocytes (Samavati et al. 2008), kidney cells, and mammary epithelial cells (Yadava et al. 2013). Other inflammatory cytokines such as interleukins (e.g., IL-1 and IL-6) also affect OxPhos via Stat3 and Mimitin (Wegrzyn et al. 2009a, b). IL-6/Stat3 signaling may play a role in tumorigenesis in specific cellular contexts (Leslie et al. 2010). TGF- β , a pleiotropic cytokine, inhibits tumor progression during the early phase of tumorigenesis, but potentiates it in later phases. Its opposing effects on the early and late phases of tumorigenesis may depend on its influence on the OxPhos system (Fosslien 2008). Other cytokines such as Wnt and leptin also suppress OxPhos (Le et al. 2012; Park et al. 2010). Therefore, chronic exposures to cytokines may promote tumorigenesis by facilitating a switch toward glycolysis by suppressing OxPhos. Increased glycolysis can upregulate NF- κ B activity in the presence of p53 suppression due to OxPhos deficiency (Compton et al. 2011; Kawauchi et al. 2008).

Lifestyles Influence OxPhos

OxPhos is susceptible to dietary influences and physical activity. High-fat diets impair OxPhos and cause obesity (Sampey et al. 2012). Obesity results in low-grade chronic inflammation, which is thought to promote tumorigenesis (Chen et al. 2010; Garcia-Ruiz et al. 2006; Harvey et al. 2011; Park et al. 2010; Remels et al. 2010). Physical activity improves mitochondrial mass and function in brain and muscle (Lanza et al. 2008; Lumini-Oliveira et al. 2011; Steiner et al. 2011). Whether balanced diets and physical activity can also improve OxPhos in mitotic tissues is unclear. They may enhance OxPhos by reducing suppressive cytokines. The OxPhos suppression could be a unifying mechanism by which dietary factors, cytokines, and physical inactivity could increase cancer risk associated with obesity (Thun et al. 2010).

Dietary factors and pharmacological drugs may also alter cellular cholesterol homeostasis. Cholesterol accumulation in mitochondrial membranes reduces their fluidity, impairs OxPhos, and increases oxidative/redox stress (Bosch et al. 2011a; Campbell and Chan 2008). The expressions of Cav-1 (caveolin-1) and TSPO (translocator protein p18) are altered in cancerous tissues. Both proteins control mitochondrial cholesterol levels (Mukherjee and Das 2012; Sloan et al. 2004; Sloan et al. 2009). Whereas Cav-1 prevents cholesterol accumulation in mitochondria by exporting it to the plasma membrane, the TSPO brings cholesterol to mitochondria (Bosch et al. 2011b; Midzak et al. 2011). Therefore, decreased Cav-1 and increased TSPO levels, as observed in cancers, would increase mitochondrial cholesterol content (Campbell and Chan 2008). The reduced mitochondrial membrane fluidity can confer apoptosis resistance. It has been found that cholesterol-lowering drugs confer apoptotic sensitivity to cancer cells (Cheng et al. 2011a). Apart from changes in membrane fluidity, an improvement in OxPhos could also be responsible because

OxPhos defects are associated with resistance to apoptotic death (Compton et al. 2011; Dey and Moraes 2000).

Effects of Toxins and Drugs on OxPhos

A large number of compounds of natural and synthetic origin can impair OxPhos (Hollerhage et al. 2009; Olszewska and Szewczyk 2013; Yadava et al. 2013b). Some of these compounds (cationic and weak acids) may accumulate several hundred fold higher in mitochondria than in cytosol because of the $\Delta\psi_m$. Their excessive accumulation in the mitochondrial matrix can impair the efficiency of OxPhos and cause oxidative/redox stress. Therefore, chronic exposure to them could cause cancer and other diseases. Some of the known respiratory chain inhibitors are natural compounds that cause disease upon chronic exposure. For example, rotenone is a phytochemical that inhibits Complex I irreversibly and it is used as a pesticide. Its chronic exposure causes Parkinson's disease (Betarbet et al. 2000). OxPhos damage induced by environmental exposures is implicated in many age-associated neurodegenerative diseases including Parkinson's and Alzheimer's disease. Because OxPhos deficiency is common to both cancer and neurodegenerative diseases, there is a growing appreciation for overlapping presentation (Hedskog et al. 2012). Environmental toxins and pharmacological drugs with mitochondrial side effects could play causative roles in cancer and other age-associated diseases by suppressing OxPhos.

Phytochemicals in Cancer Prevention and Treatment

Phytochemicals influence many aspects of cellular and whole body physiology and thereby exert their effect on the overall process of tumorigenesis. They can curb/enhance oxidative/redox stress, inhibit/promote cell proliferation, increase/decrease susceptibility to apoptosis, alter immune response, suppress/promote inflammation, and so on. Anecdotal evidence suggests that some plant extracts could be useful in preventing various types of cancers. Polyphenolics, terpenoids, glucosinolates, and anthraquinones have shown promise in cancer prevention and treatment (Herr and Buchler 2010; Keijer et al. 2011; Shirakami et al. 2012; Thomasset et al. 2007). Although the exact mechanisms for their action are unclear, these structurally different classes of phytochemicals appear to modulate redox responses and restrain inflammation and angiogenesis, the pathways important to cancer prevention (Tosetti et al. 2009). Cross-talk of the energy metabolism with different signaling pathways via oxidative/redox stress appears to be responsible for the antitumor activities of phytochemicals.

Phytochemicals can regulate oxidative stress and redox balance by influencing mitochondrial biogenesis and function. Resveratrol, curcumin,

sulforaphane, and salidroside are suggested to reduce oxidative stress (Gaona-Gaona et al., 2011; Jiang et al., 2012; Li et al., 2011; Soetikno et al., 2013). Resveratrol regulates Sirt 1 activity and reduces acetylation of PGC1 alpha, a master regulator of mitochondrial biogenesis (Lagouge et al. 2006; Momken et al. 2011). Enhanced mitochondrial biogenesis impairs carcinogenic processes (Wang and Moraes 2011). Curcumin prevents diet-induced obesity by promoting mitochondrial biogenesis and preventing Complex I function decline (Kuo et al. 2012). Salidroside found in the root extracts of the *Rhodiola rosea* may protect mitochondria under stressful conditions and preserve OxPhos (Abidov et al. 2003; Zhong et al. 2010). The activation of NRF2 (NFE2-related factor 2), a protein that controls expression of the critical antioxidant enzymes and the signaling pathways such as p53 and NF- κ B could be another key mechanism of action of phytochemicals (Niture et al. 2010; Wakabayashi et al. 2010). If chronic inflammation is proven to be a key driver of cancer development, then phytochemicals with potent anti-inflammatory activity will be useful in cancer prevention. A diet including curcumin decreases Cox-2, IL-6, and TNF α in monocytes and it curbs high-fat diet-induced metabolic syndrome that is linked with increased cancer risk (Bengmark, 2006; Cho et al., 2007; Goel et al., 2001; Jain et al., 2009; Soetikno et al., 2013). Salidroside augments adaptive immune responses and decreases the expression of cytokines related to chronic proinflammatory macrophage stimulation (Guan et al., 2011; Li et al., 2013). Given that proinflammatory cytokines can suppress OxPhos (Yadava et al. 2013), which impairs p53 activity (Compton et al. 2011), one can assume that by the nature of their ability to reduce the expression of these cytokines, phytochemicals could have chemopreventive activities.

Conclusion

Although OxPhos is a key aspect of cellular physiology, it is not essential for survival and proliferation of mammalian cells (Donnelly and Scheffler 1976). In the absence of OxPhos, cells can meet their ATP demands by upregulating glycolysis without any decline in their ability to proliferate. The upregulation of glycolysis is thought to confer proliferative advantages to cells (Ward and Thompson 2012). Altered functions of tumor suppressors and oncogenes can switch cellular metabolism toward glycolysis and suppress OxPhos. Although such alterations are secondary, the primary OxPhos deficiencies can also promote tumorigenesis (Chen et al. 2012a; Hashizume et al. 2012b). OxPhos deficiency in cancers is supported by genetic evidence and by the suppressive effects of inflammatory cytokines (Chandra and Singh 2010; Yadava et al. 2013). We propose that age-dependent decline of OxPhos may play a causative role in increased incidence of cancer. The interaction of key signaling pathways such as p53, AKT, and NF- κ B with OxPhos may play a key role in increased cancer incidence. Lifestyles and environmental factors may predispose to tumorigenesis by impairing OxPhos.

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Estrogen Receptor—Tumor Suppressor Protein p53 Signaling Crosstalk as Potential Targets of Xenoestrogens

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Abstract Excessive exposure to endogenous estrogens can lead to adverse health effects including higher risk for diseases such as breast cancer. Many exogenous chemicals have been known to affect the normal physiology of organisms including human beings. Among these are compounds that affect the endocrine system. These compounds, collectively called xenoestrogens, are either synthetic or naturally occurring in plants (phytoestrogens). The cellular effect of xenoestrogens has been studied mostly in the context of nuclear gene regulation. Although there is ample evidence of the important roles of crosstalk between signaling pathways and nuclear–mitochondrial communication in normal and pathophysiology, these have been largely ignored as potential targets for xenoestrogens. However, available evidence points to the importance of analyzing the effect of xenoestrogens from this angle for better understanding of both harmful as well as beneficial health effects of xenoestrogens including phytoestrogens.

Keywords Estrogen receptor • Tumor suppressor • p53 • Xenoestrogens • Phytoestrogens • Mitochondria • Apoptosis • Cell cycle • MicroRNA

Introduction

Communication and coordination between nuclear and mitochondrial genomes is required for the regulated expression of proteins required for mitochondrial biogenesis and function, which are essential in maintaining the fate of the cell (Leigh-Brown et al. 2010). Therefore, environmental chemicals or agents that affect nuclear function could affect mitochondrial functions and vice versa. Among these chemicals, endocrine disruptors are of major health concern as they can interfere with normal endocrine signaling in the body. As per the International Program for Chemical Safety, endocrine disruptors are “exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health

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effects in an intact organism, its progeny, or (sub)populations.” The endocrine disruptors affect normal physiology by (i) mimicking the endogenous hormones by binding to their receptors, (ii) blocking the endogenous hormone receptors from binding the endogenous hormones, and (iii) altering the synthesis and function of endogenous hormone receptors and hormones (Ropero et al. 2006). This chapter provides a brief description of how xenoestrogens affect cellular estrogen and p53 signaling that might involve both nuclear and nonnuclear compartments such as mitochondria.

Nuclear and Mitochondrial ER and p53

Synthetic and plant-derived xenoestrogens include estrogenic, antiestrogenic, and selective estrogen receptor modulator (SERM)-like compounds that are capable of interfering with genomic and nongenomic signaling (Safe and Papineni 2006; Wong and Walker 2013). Xenoestrogens are reported to mediate their biological effects by binding estrogen receptor- α (ER α) and estrogen receptor- β (ER β) and regulating their transcriptional functions. The current bioassays for xenoestrogens are based on this concept; for example, gene transcription reporter assays and ER competitive binding assays are used frequently. Therefore, although there have been several studies on the effect of xenoestrogens on the nuclear functions of ERs, very little, if any, information is available on the impact of these chemicals on signaling crosstalk involving ERs and other important proteins such as tumor suppressor p53. Moreover, the effect of these chemicals on signaling in nonnuclear locations such as mitochondria has remained largely unclear.

ERs are nuclear hormone receptors that act as transcription factors to regulate genes involved in growth, development, and differentiation of secondary sex characteristics, homeostasis, and metabolism and play a fundamental role in proliferation of breast cancer cells (Ali and Coombes 2002; Pearce and Jordan 2004; Shao and Brown 2004; Osborne and Schiff 2005).

p53 is a key tumor suppressor protein that serves as a sensor of cellular stress, and by integrating various signaling pathways, plays a central role in cellular processes such as cell cycle arrest, apoptosis, senescence, and differentiation. Since its discovery in 1979, reports have been continuously emerging on multiple functions of p53 in normal and cancer cells. In addition to its ability to initiate cell-cycle arrest and apoptosis, it has been shown to regulate metabolism, autophagy, and oxidative status of the cell (Bensaad et al. 2006; Matoba et al. 2006; Bensaad and Vousden 2007; Cheung and Vousden 2010). Of note, p53 elicits protective, prosurvival cellular responses to maintain genome integrity and viability in cells that sustain limited and reversible damage. These various responses rely on the ability of p53 to function as a transcriptional regulator of an increasing array of target genes as well as on its transcription-independent activities including those that occur in the cytosol and mitochondria.

ER and p53 Signaling Crosstalk in Normal and Cancer Cells

ER and p53 have largely opposite roles in normal and cancer cells. Estrogen receptor α (ER α) plays an important role in the onset and progression of breast cancer, whereas p53 functions as a major tumor suppressor. Most of the information about ER–p53 crosstalk in cancers comes from studies in breast cancer. In comparison to other cancers, overall frequency of p53 mutation in breast cancer is about 20%; however, wild-type p53 is functionally incapacitated. The novel mechanism by which ER α , generally upregulated in luminal breast cancer, suppresses the p53 function was discovered in our laboratory (Liu et al. 2006, 2009; Sayeed et al. 2007; Konduri et al. 2010). Consistent with this finding, clinical studies by us and others showed that ER α -positive patients expressing wild-type p53 were more responsive to tamoxifen therapy (Bergh et al. 1995; Berns et al. 2000; Miller et al. 2005; Yamashita et al. 2006; Konduri et al. 2010). Various other studies have documented the delicate relationship of estrogen signaling and ER α with p53 (Diaz-Cruz and Furth 2010; Liu et al. 2000; Duong et al. 2007; D'Assoro et al. 2008; Katayama and Sen 2011). Genetic support for this idea comes from the longstanding clinical observation that ER α -positive breast cancers express wild-type p53 whereas ER α -negative ones harbor mutant p53 (Cattoretti et al. 1988; Miller et al. 2005). These observations suggest that functional suppression of p53 is an important step in breast oncogenesis. In addition to the functional regulation by protein–protein interaction, ER α and p53 regulate each other at the transcriptional level as well. p53 has been shown to be recruited to the ER α gene promoter resulting in increased transcription of ER α (Angeloni et al. 2004; Shirley et al. 2009). On the other hand, ER α was reported to activate p53 transcription by binding to ERE half-sites within the promoter and knockdown of ER α decreases expression of p53 and its downstream targets (Berger et al. 2012). Together, these observations suggest the existence of a feedback loop between ER α and p53.

Impact of Xenoestrogens on Gene Transcription

Transcriptional signature profiles (TSPs) determined by Affymetrix microarray when breast cancer cells were proliferating at comparable rates in the presence of various estrogens showed that TSPs of cells treated with xenoestrogens were distinct from those of cells treated with 17 β -estradiol; the former strongly enhanced expression of the genes involved in mitochondrial oxidative phosphorylation, whereas the latter showed minimal effects (Shioda et al. 2006). Treatment of progenitor-containing mammospheres with xenoestrogen diethylstilbestrol downregulated *microRNA-9-3* (*miR-9-3*) and there was aberrant methylation of its promoter. Intriguingly, *miR-9-3* is involved in the p53-dependent apoptotic pathway. Therefore, epigenetic silencing of *miR-9-3* in the nucleus promoted cell proliferation by inhibiting apoptosis

(Hsu et al. 2009). Another target of inhibition by diethylstilbestrol is *miRNA-34b* (*mir-34b*). The inhibition of *mir-34b* resulted in restoration of the protein levels of the *mir-34b* targets cyclin D1 (*Ccnd1*) and *Jag1* in MCF-7 cells (Lee et al. 2011). It is important to note that the inhibition of *mir-34b* was observed only in cells expressing both ER α and wild-type p53 and treatment with 17 β -estradiol disrupted binding of p53 to the promoter. Furthermore, xenoestrogens have been reported to regulate the activity of arginine methyl transferases (PRMTs; Cheng and Bedford 2011). PRMTs are important transcriptional coregulators of various proteins including ER and p53 (Jansson et al. 2008; Le Romancer et al. 2008).

It has been reported that dichlorodiphenyltrichloethane (DDT) exposure activated ER α in mouse liver leading to increased expression of target genes including *Cyp2b10*, *Gadd45 β* , *cMyc*, *Mdm2*, *Ccnd1*, *CDK4*, and *E2F1*. In addition, DDT exposure increased Rb phosphorylation, and decrease in p53 protein level and transcriptional activity. Consistent with these observations, there was higher expression of proteins mediating increased cell-cycle progression and decreased apoptosis (Kazantseva et al. 2013).

Conclusion and Future Perspectives

The reports mentioned above, although few in number, are suggestive of the importance of nuclear–mitochondrial communication and ER and p53 signaling as targets of xenoestrogens in mediating their multiple cellular effects leading to diseases such as cancer. The vast majority of investigations done on tumor development have focused on events in understanding the molecular and genetic hallmarks of the disease (Hanahan and Weinberg 2011). There is increasing evidence of the important role of mitochondria, bioenergetics, and metabolism in the pathophysiology of various diseases including cancer (Wallace 2013). Therefore, the importance of analyzing the effects of xenoestrogens on ER and p53 signaling in the nucleus and mitochondria cannot be overstated. Future research will unravel important information in this exciting area.

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Mitochondrial Regulation of Cell-Death

Richard Jäger and Howard O. Fearnhead

Abstract Mitochondria are much studied organelles, investigated for decades for their vital role as powerhouses of the cell and for the evolutionary history concealed in the mitochondrial genome. To this day, research on mitochondria is still yielding fascinating new information. Over the past two decades a large body of evidence has been accumulated showing these structures, which are at the heart of metabolic processes sustaining life, at same time containing the seeds of cellular self-destruction. This chapter describes the death-inducing proteins that are released from mitochondria, the mechanisms of release, as well as how release is regulated. Finally, phytochemicals that affect the release of lethal mitochondrial proteins are discussed.

Keywords Programmed cell death • Mitochondrial apoptogens • Bcl-2 family • Mitochondrial outer membrane permeabilization (MOMP)

Introduction

The role of mitochondria in cell death processes has been studied for many years. Early studies focused on mitochondria as the target for various toxicants that inhibited mitochondrial function, thus leading to cell death. For example, rotenone inhibits electron transport, blocking oxidative phosphorylation, and thus causing death. Only later did it become clear that the mitochondria played an active role in cell death and that release of mitochondrial proteins is a defining event in cell death induced by a wide range of physiological and pharmacological stimuli. Some of the first evidence that hinted at the importance of mitochondria in cell death was the discovery that Bcl-2, a protein that blocks apoptotic cell death is associated with the mitochondrial membrane (Hockenberry 1990). A reduction of mitochondrial membrane potential was subsequently identified as an early event in apoptotic cell death

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(Vayssiere et al. 1994; Zamzami et al. 1995a, b). Cell-free systems of apoptosis also played their part with the demonstration that apoptotic changes in *Xenopus* egg nuclei require a fraction containing mitochondria (Newmeyer et al. 1994) and that caspase activation in extracts from HeLa cells required the mitochondrial protein cytochrome *c* (Liu et al. 1996). The discovery that cytochrome *c* escaped from mitochondria during cell death and that this was blocked by Bcl-2 firmly established the idea that mitochondria actively participate in cell death by releasing molecules and that this release was regulated. What has followed over the last 15 or so years is a frenzy of research activity that has identified a group of mitochondrial proteins that kill cells when released and at the same time uncovered much of the molecular mechanism that regulates their release.

In this chapter we describe the death-inducing proteins that are released from mitochondria. We then consider the mechanisms that release these death-inducing mitochondrial proteins as well as how release is regulated. Finally, we briefly discuss how phytochemicals may affect the release of these mitochondrial proteins, an area that is explored in more detail in other chapters.

Apoptogens

Central to the role of mitochondria in regulating cell death is the release of proteins normally sequestered behind one or both of the mitochondrial membranes. This release can often result in a loss of mitochondrial function, an event normally incompatible with cell survival. However, the released proteins are also free to interact with new partners in the cytoplasm and nucleus with dramatic consequences for the cell. It is now well established that the released proteins per se initiate cell death and that mitochondrial dysfunction rather represents an epiphenomenon of the associated mitochondrial membrane perturbations. The key mediators of cell death released from the mitochondria are all encoded by nuclear genes and are: cytochrome *c*, second mitochondrial activator of caspases/direct inhibitor of apoptosis protein-binding protein with low pI (SMAC/DIABLO), Omi/HtrA2, apoptosis-inducing factor (AIF), and endonuclease G (Fig. 1). These factors were discovered using a range of different experimental approaches and model systems (Table 1). Some of them are central to the activation and activity of caspases, whereas others induce caspase-independent cell death.

Cytochrome *c* (CYCS) Cytochrome *c* is a small (12 kDa) globular protein found between the inner and outer mitochondrial membranes that plays a central role in oxidative phosphorylation, transferring electrons between Complex III and Complex IV. As a nuclear encoded gene, it is translated in the cytoplasm to produce the *apo* form of the protein (lacking heme). The *apo* form lacks any clear mitochondrial localization signal, but none the less it is imported by the TOM complex. Coincident with import, the *apo* protein is conjugated to heme by heme lyase to form the

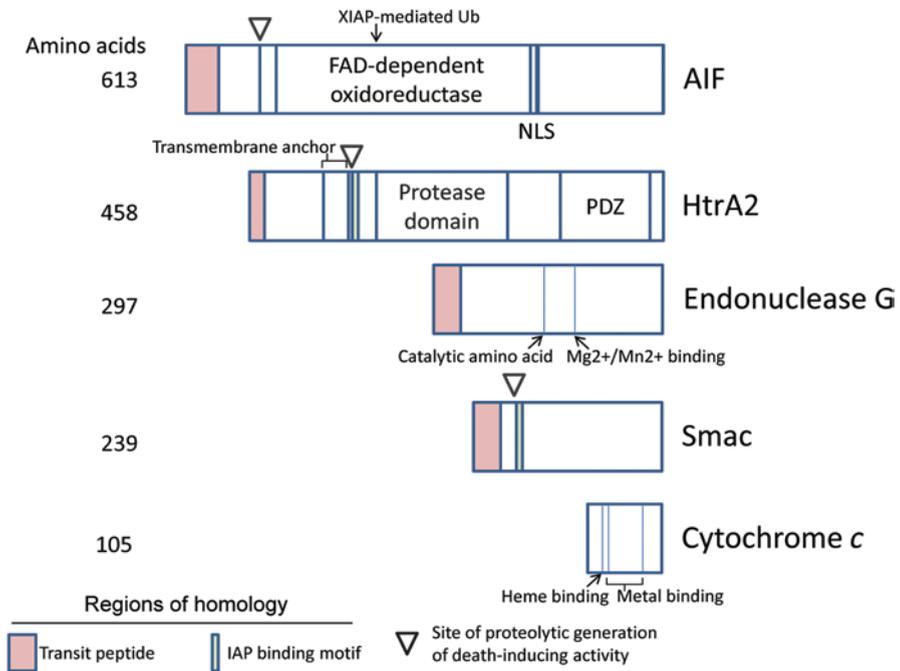


Fig. 1 Schematic showing prodeath proteins released from mitochondria. Domain structures and amino acids important to the normal and prodeath activities of these proteins are indicated. The N-terminals of AIF, Smac, HtrA2, and Endonuclease G all contain transit peptides important for mitochondrial import. Smac and HtrA also possess an IAP-binding domain homologous to the N-terminal of the caspase-9 p10 subunit

holo-protein which is the form that participates in electron transfer. During mitochondrial outer membrane permeabilization (MOMP, discussed in detail later in this chapter) cytochrome *c* is released into the cytosol, allowing it to interact with a 150 kDa protein, Apaf-1 (Zou et al. 1997). This interaction is a key event in triggering the formation of a large protein complex called the apoptosome that induces cell death by activating caspases (Cain et al. 2002). The cytochrome *c* binding site on Apaf-1 is not clearly identified, although some evidence suggests that it lies within the C-terminal WD40 repeats of Apaf-1 (Benedict et al. 2000 Hu et al. 1998). Several lines of evidence suggest that lysine 72 in cytochrome *c* is a key amino acid for the Apaf-1 interaction (Yu et al. 2001). It is important to note that although the apo form can bind Apaf-1, it cannot induce apoptosome formation (Martin and Fearnhead, 2002; Martin et al. 2004), thus the addition of heme is essential for both the prolife and prodeath activity of cytochrome *c*. Apaf-1 is not the only cytochrome *c* interactor relevant for cell survival and death. The binding of both ATP and tRNA to cytochrome *c* have been shown to be important determinants that regulate apoptosome formation (Chandra et al. 2006; Mei et al. 2010).

Table 1 Prodeath proteins released from mitochondria, their normal function in living cells, the activity associated with cell death, and the methods used in their discovery

Protein	Nondeath Activity/ Function	Death Activity/ Function	Identified by
Cyt <i>c</i>	Electron transport in oxidative phosphorylation	Apoptosome formation through binding to Apaf-1	Biochemical purification using cell-free system of caspase activation (Li et al. 1997)
SMAC	Uncertain	Inactivation of XIAP and other IAPs through binding	Biochemical purification using cell-free system of caspase activation (Du et al. 2000). Affinity purification to identify XIAP binding proteins (Verhagen et al. 2000)
HTRA2	Protease	Inactivation of XIAP and proteolysis of other substrates	Affinity chromatography to identify XIAP binding proteins (Hegde et al. 2002)
AIF	Oxidoreductase	Induction of HMW DNA fragmentation through interaction with other proteins	Biochemical purification using cell-free system of chromatin condensation (Susin et al. 1999)
Endo-G	Nuclease: Mitochondrial DNA replication	Nuclease: Induction of oligonucleosomal DNA fragmentation	Biochemical purification using cell-free system (Li et al. 2001)
ARTS	Uncertain	XIAP-inactivation	Retroviral insertional mutagenesis (Larisch et al. 2000)
Bit1	tRNA hydrolase	Caspase-independent cell death	cDNA library screening (Jan et al. 2004)
79 other proteins	Varied	Uncertain roles in cell death	Proteomic methodology* using isolated mitochondria treated with atractyloside (Patterson et al. 2000)

Cyt *c* = Cytochrome *c*; Endo-G = Endonuclease-G.

The essential role of cytochrome *c* in the mitochondrial electron transfer chain has limited the usefulness of knockout animals as cytochrome *c* null mutants show reduced size, delayed development, and die in utero at embryonic day 8.5 (Li et al. 2000). To overcome this problem, mice expressing a K72A mutant cytochrome *c* have been made (Hao et al. 2005). This mutation does not affect electron transfer but does abolish apoptosome formation and reduce caspase activation. The phenotype of these mice resembles that of Apaf-1, caspase-9, and caspase-3 null mice (the other components of the apoptosome), strongly supporting the cell-death role of cytochrome *c* revealed by biochemical studies.

Cytochrome *c* is a highly conserved gene across species and it has long been used to investigate evolutionary processes. The conservation of cytochrome *c*-dependent caspase activation has also been investigated, revealing that the cytochrome *c*/Apaf-1/caspase module is present in a wide range of metazoans, from planaria to humans, arguing that this is an evolutionarily old mechanism (Bender et al. 2012). However, although programmed cell death in yeast involves re-

lease of mitochondrial proteins, including cytochrome *c*, yeast does not express a homologue of either Apaf-1 or caspases. Nor is yeast cytochrome *c* able to substitute for mammalian cytochrome *c* in caspase activation, in part because of tri-methylation at K72 (Chertkova et al. 2008; Sharonov et al. 2005; Yu et al. 2001). It is not clear whether cytochrome *c* release can play a role in regulating yeast cell death in the absence of Apaf-1, or whether this is a bystander event with cell death being induced by other mitochondrial proteins such as the yeast homologues of AIF and Endonuclease G (see below).

Second Mitochondrial Activator of Caspases (SMAC; DIABLO) SMAC/DIABLO (here called Smac) is, like cytochrome *c*, a nuclear gene that encodes a protein found between the inner and outer mitochondrial membranes (Du et al. 2000; Verhagen et al. 2000). Although its normal role within mitochondria is not as clear as it is for cytochrome *c*, once released Smac promotes caspase activity thus killing cells. Smac is synthesized as a 239 amino acid (27 kDa) proform with a 55 amino acid N-terminal domain that contains a mitochondrial targeting sequence. The proform undergoes proteolytic maturation upon import to generate a form capable of promoting caspase activity. The new N-terminus is essential for its ability to promote caspase activity and a seven amino acid peptide mimicking the N-terminus is sufficient to activate caspases *in vitro*.

The mechanism by which Smac regulates caspase activity is well understood and involves the binding of Smac to the X-linked inhibitor of apoptosis (XIAP; Chai et al. 2000; Liu et al. 2000; Srinivasula et al. 2000). XIAP is a potent caspase-9 and -3 inhibitor that binds to the N-terminal of the small (p10) subunit of an active caspase. The binding of XIAP to Smac is mediated by the N-terminal amino acids of Smac and by binding to XIAP, Smac alleviates XIAP-mediated caspase inhibition and promotes cell death.

XIAP belongs to the inhibitor of apoptosis protein family (IAPs) that also contains cIAP-1 and cIAP-2 (Salvesen and Duckett 2002). Many IAP proteins, including XIAP, c-IAP1, and c-IAP2, contain a carboxyl-terminal RING domain that functions as an E3 ubiquitin ligase. In the current model, IAP auto-ubiquitylation regulates the levels of these proteins and binding to Smac or small molecule Smac-mimetics increases auto-ubiquitylation, destabilizing the proteins and causing their concentration in the cell to fall.

c-IAP1 and c-IAP2 associate with the type 2 TNF receptor through binding to TNF receptor-associated factors, which inhibits the ubiquitylation of c-IAP1, stabilizing the protein and therefore decreasing apoptosis (Csomos et al. 2009). Although cIAPs bind to caspases, they do not inhibit protease activity, instead the antiapoptotic activity of cIAP1 occurs through sequestration of Smac, preventing Smac from binding to XIAP. cIAP also regulates necroptotic cell death by blocking activation of the RIP1 and RIP3 kinases, inhibition that is alleviated by Smac mimetics (McComb et al. 2012). Thus, the release of Smac and Omi may potentially regulate this form of cell death, although the release of these proteins during necroptosis has not been demonstrated.

Targeted deletion of Smac in mice did not elicit any phenotypical alterations, and also neither survival *in vitro* of activated B- and T-cells or fibroblasts derived from these mice were affected, suggesting the lack of a role of Smac in inducing cell

death or redundant mechanisms (Okada et al. 2002). A subsequent study, however, revealed a role of Smac in executing cell death when caspase-3 was absent: Smac/caspase-3 double-deficient mice showed perinatal lethality, and fibroblasts derived from such mice displayed increased survival selectively upon stimulating the intrinsic apoptotic pathway (Hui et al. 2011).

Htra2 (OMI) Htra2 is a protease expressed as a 49 kDa proform that is targeted to the inner membrane space of the mitochondria (Suzuki et al. 2001; van Loo et al. 2002). It belongs to a large family of high temperature requirement serine proteases whose members are characterized by the presence of a trypsin like protease domain and one or two C-terminal PDZ-domains. HtrA proteases are found in nearly all prokaryotic and eukaryotic genomes and they play a key role in protein quality control by cleaving unfolded proteins and thus protecting mitochondria from the accumulation of damaged/misfolded proteins (Clausen et al. 2011). In addition, HtrA proteases can also cleave some folded proteins and therefore regulate various signaling processes (Vande Walle et al. 2008). It is this latter role in regulating signaling processes that appears important for the cell death-inducing activity of HtrA2 (Suzuki et al. 2004).

Following import into mitochondria, HtrA2 undergoes proteolytic maturation by a poorly understood mechanism. Autocatalytic cleavage of HtrA2 generates a new N-terminal that resembles the N-terminal of Smac (Seong et al. 2004). Thus, the release of HtrA2 into the cytosol allows interaction with XIAP and thus increases caspase activity by alleviating XIAP-mediated caspase inhibition (Li et al. 2002). IAPs are also substrates for HtrA2 (Srinivasula et al. 2003; Yang et al. 2003), and binding is accompanied by IAP degradation and the subsequent fall in IAP concentration contributes to the proapoptotic effect of HtrA2. The degradation of XIAP by HtrA2 is augmented by the binding of HtrA2 to an interferon-regulated protein, GRIM19 (Ma et al. 2007).

HtrA2 can also regulate caspase-independent cell death as the overexpression of a mutant HtrA2 lacking the IAP binding domain induced cell death and HtrA2 induces cell death in the absence of Apaf-1 and caspase-9 (Hegde et al. 2002) or in the presence of pharmacological caspase inhibitors (Suzuki et al. 2001). It is important that a proteolytically inactive mutant does not induce cell death indicating that cleavage of one or several proteins is required (Suzuki et al. 2001; Verhagen et al. 2002). Proteomic analysis and other approaches have identified a panel of HtrA2 substrates (Table 2) (Trencia et al. 2004; Vande Walle et al. 2007; Vande Walle et al. 2010). How cleavage of these proteins contributes to cell death and whether there are as yet unidentified substrates remains to be determined.

Contrary to expectations derived from the overexpression approaches in cell culture, the targeted deletion of HtrA2 in mice did not reveal deficiencies in cell death, but rather increased loss of cells, particularly of neurons, leading to the development of Parkinson-like neurodegenerative disease (Martins et al. 2004). Loss of HtrA2 led to an altered mitochondrial ultrastructure and mitochondrial dysfunction, the latter caused by mitochondrial uncoupling and ATP depletion (Plun-Favreau et al. 2012). As HtrA2/Smac double-deficient mice did not display an additional phenotype, the protease function of HtrA2, and not deficient XIAP inactivation,

Table 2 Known HtrA2 substrates

Protein Cleaved	Symbol	Function	Reference
Tubulin	α , β 2, β 4, β 6	Cytoskeleton	(Vande Walle et al. 2007)
Actin		Cytoskeleton	(Vande Walle et al. 2007)
Vimentin		Cytoskeleton	(Vande Walle et al. 2007)
Retinal dehydrogenase	RALDH2		(Vande Walle et al. 2007)
Hydroxyacyl-coenzyme A dehydrogenase type II	HADH2		(Vande Walle et al. 2007)
Elongation factor-1a	EF-1 α	Translation	(Vande Walle et al. 2007)
Eukaryotic Initiation factor 4G1	EIF-4G1	Translation	(Vande Walle et al. 2007)
Transcription Intermediary factor-1b	TIF-1 β	Transcription	(Vande Walle et al. 2007)
Vacuolar sorting protein 4b	VSPS4B	Intracellular protein trafficking	(Vande Walle et al. 2007)
Carbamoyl-phosphate synthetase aspartate transcarbamoylase-dihydroorotase	CAD	de novo pyrimidine synthesis	(Vande Walle et al. 2007)
	KIAA1967	Deleted in breast cancer-1 or DBC-1?	(Vande Walle et al. 2007)
	KIAA0251	Decarboxylase domain?	(Vande Walle et al. 2007)
β -Amyloid precursor protein	APP		(Park et al. 2006)
	Ped/PEA15	Antiapoptotic protein	(Trencia et al. 2004)
	WARTS	Cell-cycle regulator	(Kuninaka et al. 2007)
	HAX-1		(Cilenti et al. 2004)
X-linked inhibitor of Apoptosis	XIAP	Caspase-inhibitor, E3-ligase	(Srinivasula et al. 2003; Yang et al. 2003)
Cellular inhibitor of Apoptosis-1	cIAP-1	Cell-death regulator, E3-ligase	(Srinivasula et al. 2003; Yang et al. 2003)
Cellular inhibitor of Apoptosis-2	cIAP-2	Cell-death regulator, E3-ligase	(Srinivasula et al. 2003; Yang et al. 2003)
	HtrA2	IAP-inhibitor, Serine protease	(Savopoulos et al. 2000; Seong et al. 2004)

seems to account for the observed phenotypes, and HtrA2 does not seem to play an essential role in cell death induction (Martins et al. 2004).

Apoptosis Inducing Factor (AIFM1; PDCD8; COXPD6) AIF is a flavoprotein found between the inner and outer mitochondrial membranes with a key role in cas-

pase-independent cell death (Cande et al. 2002). AIF possesses three recognizable domains: an N-terminal signal sequence that is removed after proper localization in mitochondria, a spacer sequence, and finally an oxidoreductase domain. The 613 amino acid proform is imported into mitochondria and undergoes proteolysis to remove the first 54 amino acids of the N-terminus. The import of AIF into mitochondria is poorly understood (Chiang et al. 2012). In the mitochondria AIF is anchored in the inner membrane and functions as an FAD-dependent oxidoreductase with a vital role in oxidative phosphorylation (Vahsen et al. 2004). In response to apoptotic stimuli AIF is further cleaved at amino acid 101 to a 57 kDa prodeath form in a calcium-dependent fashion (Norberg et al. 2008) and after its release from mitochondria, the truncated AIF translocates to the nucleus. This movement is regulated by Hsp70 and Cyclophilin A (Zhu et al. 2007). Once in the nucleus, it induces caspase-independent chromatin condensation and DNA fragmentation to 10–50 kbp fragments, which represent an early or intermediate step in apoptotic DNA fragmentation (Cohen et al. 1994) that culminates in internucleosomal DNA cleavage (Susin et al. 1999). DNA binding of AIF is necessary for its prodeath activity (Ye et al. 2002), however, AIF also binds Cyclophilin A resulting in augmented DNase activity (Cande et al. 2004) and AIF binds to histone 2AX (Artus et al. 2010). In the cytosol AIF is subject to regulation by XIAP, which both binds and ubiquitinylates AIF, inhibiting its ability to induce DNA fragmentation (Lewis et al. 2011; Wilkinson et al. 2008). XIAP-mediated ubiquitylation does not destabilize AIF, however, another E3 ligase, CHIP, has been identified that does mark AIF for proteasomal degradation (Oh et al. 2011).

The oxidoreductase domain of AIF shows strong homology to other oxidoreductases from vertebrates to archeabacteria. Note that key amino acids necessary for oxidoreductase activity are conserved, revealing the importance of this activity for the non-cell death roles of AIF (Vahsen et al. 2004). In addition, the AIF homologue from a slime mold, *Dictyostelium discoideum*, plays a very similar role in programmed cell death in this organism (Arnoult et al. 2001) and programmed cell death in *Saccharomyces cerevisiae* involves the release of the yeast homologue from mitochondria (Wissing et al. 2004). Together these observations suggest that the prosurvival and prodeath functions of this protein have been conserved in highly divergent species and that AIF represents a phylogenetically ancient cell-death effector (Lorenzo et al. 1999).

The Harlequin (Hq) mutation in mice results from a proviral insertion into the AIF gene and reduces AIF expression by approximately 80% (Klein et al. 2002). These mice display increased neuronal cell death that is due to an enhanced susceptibility to oxidative stress, and suggest primarily a prosurvival role of AIF. However, as neurons of Hq mice are more resistant towards neuronal excitotoxicity than wild-type mice, AIF seems to play a prodeath role, too (Cheung et al. 2005). Attempts to generate AIF knockout mice were not successful, however, AIF^{-/-} mouse embryonic stem cells were resistant to cell death upon serum deprivation and embryoid bodies derived from these cells did not display normal cavitation, suggesting roles of AIF in developmental programmed cell death (Joza et al. 2001).

Endonuclease G Endonuclease G is another nuclear encoded gene whose product is imported into mitochondria. Its normal function is thought to be in the production of primers for synthesis of mitochondrial DNA, and it is therefore localized to the mitochondrial matrix. The role of endonuclease G in cell death was uncovered using a biochemical approach to identify nuclease activities released from mitochondria (Li et al. 2001). Endonuclease G also shows activity against RNA, and it cleaves RNA more efficiently than DNA leading to the suggestion that its major role is as an apoptotic RNase rather than a DNase (Kalinowska et al. 2005). Its role in cell death appears to be conserved (Parrish et al. 2001), although endonuclease G null mice are viable and develop normally with no obvious abnormalities (Zhang et al. 2003a). Moreover, fibroblasts derived from null animals do not display any defects in apoptosis. Together these observations suggest if endonuclease G is really an apoptotic DNase, then it plays a redundant role with CAD/DFF40 in generating the internucleosomal DNA fragmentation typical of apoptosis.

Other Mitochondrial Proteins Released During Cell-Death ARTS is a septin-like protein that regulates apoptosis (Larisch et al. 2000). It is released from mitochondria and once in the cytosol it binds to XIAP (Gottfried et al. 2004). This binding is through sequences within the BIR3 domain which are different from XIAP-Smac or XIAP-HtrA2 interaction motifs (Bornstein et al. 2011; Reingewertz et al. 2011). Perhaps most provocative is the report that ARTS is released before other apoptogenic proteins to inactivate XIAP and promote later, cytochrome c-dependent events (Edison et al. 2012). How general this phenomenon is and how differential release of mitochondrial proteins is achieved is unknown. Bit1 is a peptidyl-tRNA hydrolase identified in a screen for proteins that play a role in anoikis. When released from mitochondria Bit1 induces cell death (Jan et al. 2004) at least in part through its regulation of ERK activity (Kairouz-Wahbe et al. 2008). There is evidence from ARTS knockout mice for an *in vivo* role of ARTS in cell death of hematopoietic stem and progenitor cells. This role for ARTS seems to depend on its ability to inactivate XIAP as the increased cell survival observed when ARTS is deleted is abolished when both ARTS and XIAP are deleted together (Garcia-Fernandez et al. 2010).

It is also possible that mitochondrial proteins regulating cell death are as yet unidentified and several groups have applied proteomic methods to address this question. A recent proteomic approach identified more than 70 proteins released from isolated mitochondria treated with atractyloside (Patterson et al. 2000), although the function of these proteins in cell death was not addressed. Atractyloside, a diterpenoid glucoside produced by a thistle *Atractylis gummifera* (Daniele et al. 2005), binds to the adenine nucleotide transporter, leading to mitochondrial permeability transition (mPT), mitochondrial swelling, and outer membrane rupture. Although mPT can release apoptogenic proteins, there is some controversy over whether mPT causes apoptotic or necrotic cell death (see section on MOMP below). Others have used tBid to trigger the release of Smac from isolated mitochondria (van Loo et al. 2002). Approaches using tBid seem better suited to find novel apoptogens than atractyloside as tBid represents a bona-fide part of the death machinery, although the efficacy of atractyloside demonstrates that phytochemicals can induce cell death by interfering with this machinery. Apart from the known apoptogens these authors identified proteins that included ADK2, fatty acid binding protein, a polypyrimidine tract-binding

protein, an RNA-binding protein, and acyl CoA-binding protein. The role (if any) that these proteins play in cell death is unclear.

An Integrated View of Apoptogens A very large body of information on cell-death regulators from mitochondria has been generated over the years from a wide range of different experimental systems. Despite some inconsistencies between studies there is nonetheless strong evidence supporting some central ideas.

The cytoplasmic protein XIAP emerges as an important node in the network of protein–protein interactions (Fig. 2). XIAP counteracts the prodeath activities of two mitochondrial proteins, cytochrome *c* and AIF, and thus plays a key role in regulating caspase-dependent and -independent cell death. XIAP itself is subject to regulation by mitochondrial proteins, Smac and HtrA2, which antagonize its prosurvival activity. Smac and HtrA2 also bind cIAP1, linking this IAP into the network. Although cIAP1 does not inhibit caspase-3 or -9 nor bind AIF, it can indirectly affect cell death by competing with XIAP for Smac and HtrA2. Thus, the outcome of mitochondrial release will be affected by the concentrations of both IAPs. Moreover, mitochondrial release will affect the concentration of both IAPs and Smac binding increases their auto-ubiquitinylation and destabilizes them.

cIAP1 is also an important regulator of cell death through its ability to interact with death receptor complexes and affect downstream prosurvival signaling. Activation of the TNF α receptor leads to the formation of a multiprotein complex that includes TRADD, TRAF2, RIP1, and cIAP. cIAP1-mediated ubiquitinylation of RIP1 triggers the recruitment of an additional protein, NEMO. Once recruited, NEMO activates the NF- κ B pathway which is generally considered to be antiapoptotic/prosurvival (Gyrd-Hansen et al. 2008; Haas et al. 2009).

cIAP1 also occupies an important position in the necroptotic pathway, where it inhibits RIP1 and RIP3 kinases, thus preventing necroptosis. Small molecule Smac mimetics prevent this inhibition and are often used in conjunction with other stimuli to induce necroptosis. However, whether Smac itself regulates necroptosis is uncertain. Thus, although necroptosis regulators are found in a network of interactions with mitochondrial death inducers, there is as yet no demonstrated link between the release of mitochondrial proteins and this form of cell death. Indeed, DNA-damage-induced necroptosis occurs without mitochondrial involvement (Tenev et al. 2011).

Many of the proteins released from mitochondria including Endonuclease G (Kalinowska et al. 2005), AIF (Gurbuxani et al. 2003), and cytochrome *c* (Bruey et al. 2000) have also been reported to bind to members of the heatshock proteins (HSP). Typically the interaction with HSP blocks the prodeath activity of the released protein. However, the association with HSPs has typically been detected in cell extracts and it is possible that these interactions are artifacts induced by cell lysis.

Proteins released from mitochondria have nonprotein binding partners too. Endonuclease G and AIF interact with DNA and RNA and mediate their hydrolysis, either directly or through interactions with accessory proteins (Kalinowska et al. 2005). However, cytochrome *c* also binds to tRNA and this interaction inhibits apoptosome formation by preventing cytochrome *c* from binding to Apaf-1 (Mei et al. 2010). It is unclear whether this is an example of apoptosome regulation as tRNA levels are typically high in cells. However, at least one other protein released

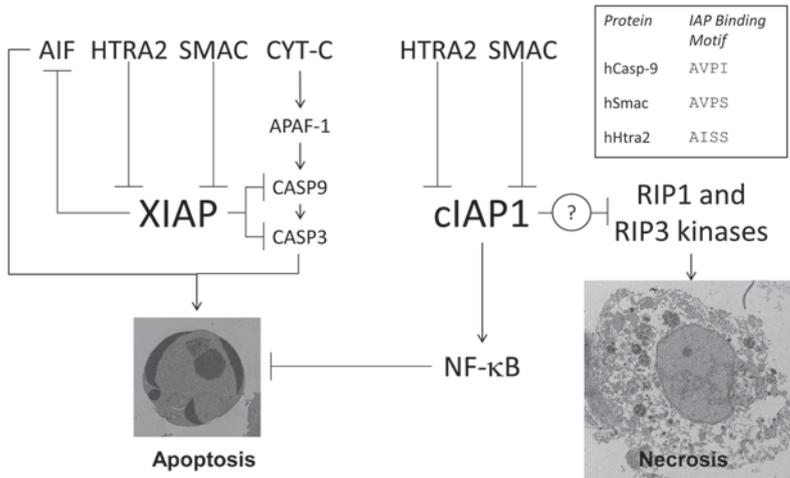


Fig. 2 Mitochondrial regulation of IAPs and cell death

from mitochondria is a tRNA hydrolase (Kairouz-Wahbe et al. 2008) therefore it is tempting to speculate that degradation of tRNA by mitochondrial proteins is a necessary step to overcome inhibition of apoptosome formation. Cytochrome *c* binding to Apaf-1 is also blocked by ATP (Chandra et al. 2006; Samali et al. 2007). ATP-binding to cytochrome *c* occurs at physiologically relevant concentrations and the metabolic status of the cell can therefore contribute to the decision to live or die by affecting whether apoptosome formation occurs.

In summary of this section on apoptogens, mitochondria contain several cell-death-inducing proteins whose sequestration is necessary for cell survival. These proteins are expressed as proproteins that do not induce cell death and that are only made competent after their import into the mitochondria. Once released, each of these death-inducing factor sets entrains a cascade of events that reach out to affect other cellular compartments, most notably the nucleus. These inducers are capable of regulating different cell-death modalities such as caspase-dependent and -independent apoptosis. The IAPs also emerge as important regulators of cell death as they directly or indirectly either regulate or are regulated by the proapoptotic proteins released from the mitochondria (Fig. 2). The consequence is that death signals or stimuli that affect mitochondria very often induce parallel and redundant pathways leading to cell death, making mitochondrial events a “point-of-no-return” in cell death processes. Thus, mitochondria, through their ability to sequester a cocktail of lethal factors and then release these factors on command, represent a key node in the signal transduction pathways regulating cell death and survival.

Mechanisms and Regulation of Release of Apoptogens

Are Apoptogens Coordinately Released? Several mechanisms have been proposed for the release of apoptogenic factors from mitochondria of cells initiating apoptosis. A common feature of those is the sudden increase in permeability of the mitochondrial outer membrane (MOM) a process called mitochondrial outer membrane permeabilization (MOMP). MOMP has been extensively studied using cytochrome *c* as the prototype apoptogen, although it is not a priori clear whether other apoptogens employ the same release mechanism. In fact, kinetic analyses revealed that AIF and EndoG are released later and by a mechanism other than cytochrome *c*, Smac, and Omi/HTRA2 which appear to be coordinately released (Arnoult et al. 2003a; Munoz-Pinedo et al. 2006).

There are studies to suggest that cytochrome *c* might indirectly affect the release of other apoptogens from mitochondria. Delivering exogenous cytochrome *c* into the cytoplasm of mammalian cells by either pinocytosis or electroporation triggers caspase activation and cell death (Chertkova et al. 2008; Gabriel et al. 2003; Gilmore et al. 2001; Sharonov et al. 2005). However, it can also trigger the release of apoptogens such as AIF and Smac in a caspase-dependent fashion (Arnoult et al. 2003b; Gabriel et al. 2003; Gilmore et al. 2001). These data suggest that in at least some cell types there are feedback mechanisms ensuring that the release of cytochrome *c* is coupled with the release of other apoptogens.

One important finding is that the release of cytochrome *c* from mitochondria involves a two-step process (Ott et al. 2002). The majority of cytochrome *c* is firmly attached to phospholipids of the inner mitochondrial membrane, mainly to cardiolipin. So this interaction must be disrupted first to generate a soluble pool of cytochrome *c* that subsequently can escape from the intermembrane space through the permeated MOM. Interestingly, during apoptosis cytochrome *c* itself peroxidizes cardiolipin to allow for its detachment, and cardiolipin peroxidation turned out to be required for release of Smac/Diablo as well (Kagan et al. 2005). Of note, in specific cell types, and upon particular death stimuli, various distinct mechanisms of cytochrome *c* release can be operating (see Gogvadze et al. 2006 for review).

Whatever the release mechanism, it is clear that the liberation of cytochrome *c* during apoptosis is controlled by members of the Bcl-2 family of proteins (Kluck et al. 1997; Yang et al. 1997).

Cell Death Signals Converge on Mitochondria via the Bcl-2 Family Proteins of the Bcl-2 family are characterized by Bcl-2 homology (BH) domains which serve for their physical interaction (see Youle and Strasser, (2008) for a review on the Bcl-2 family). They can be subdivided into multidomain proteins that are either antiapoptotic and contain four BH domains, such as the prototype member Bcl-2 or Bcl-X_L, or multidomain proteins that lack the fourth BH domain and are proapoptotic, such as Bax and Bak. The latter have been shown to stimulate cytochrome *c* release after their activation (Narita et al. 1998; Rosse et al. 1998). Bak resides permanently at the MOM whereas Bax, normally cytoplasmic, changes conformation upon activation and then inserts into the MOM. Antiapoptotic family members also localize to the MOM where they prevent Bax or Bak from stimulating cytochrome

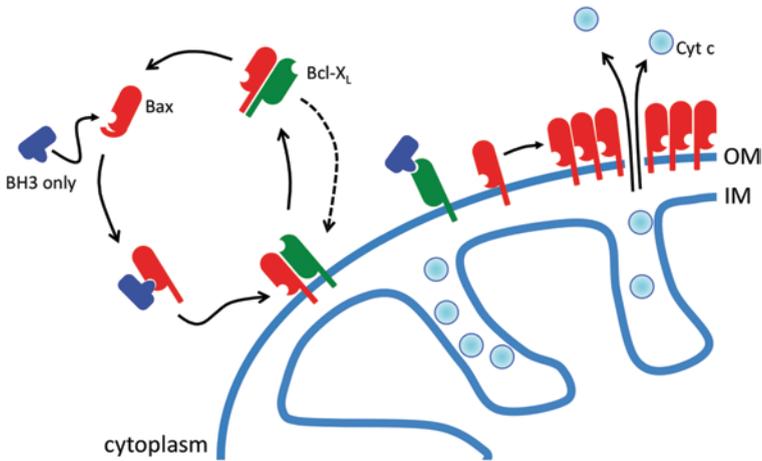


Fig. 3 Model of Bax activation and pore formation. When activated by BH3-only proteins, Bax exposes a hydrophobic tail and inserts into the outer mitochondrial membrane (OM). Normally, OM-inserted Bax is complexed by antiapoptotic Bcl-2 proteins (Bcl-XL) residing on the OM and retrotranslocated to the cytoplasm (ref: Edlich 2011, Cell 145:104). When Bcl-XL is blocked by BH3 only proteins, retrotranslocation is prevented and Bax can form large oligomeric pores through which cytochrome *c* (Cyt *c*) is released from the intermembrane space between OM and inner membrane (IM)

c release. Recently it was shown that one mechanism used by antiapoptotic Bcl-2 family members to counteract Bax is by mediating its retranslocation from mitochondria to the cytoplasm (Fig. 3, Edlich et al. 2011). A unified model of interaction of Bcl-2 family proteins was posited that integrates two proposed mechanisms of how antiapoptotic family members exert their prosurvival activity: in a less efficient manner by sequestering BH3-only proteins (see below), and in a more effective way by directly interacting with activated Bax or Bak (Llambi et al. 2011, and references therein). Multidomain Bcl-2 family members can also localize to nuclear envelope and ER where they can undergo conformational changes, but their functions are much less clear (Kim et al. 2004).

A third proapoptotic subfamily comprises those Bcl-2 family members that harbor only the third BH domain (BH3-only proteins, such as Bim or Bid). They are induced or activated by apoptotic stimuli (see below), interacting with and so transducing the signal to the anti- or proapoptotic multidomain family members, which serve as their receptors. Their binding functionally blocks the antiapoptotic multidomain family members and activates the proapoptotic members Bax or Bak by triggering conformational changes that lead to their oligomerization in the mitochondrial membrane (see Fig. 3, Llambi et al. 2011). Thus, these BH3-only proteins integrate several cellular death pathways at the level of mitochondria (see Fig. 4). Some examples are discussed below.

DNA Damage and Cell-Cycle Perturbations The fidelity of the genome is vital for an organism's survival and proper functioning. However, it is susceptible to toxic insults, such as DNA damaging agents or irradiation. After DNA damage, cells either

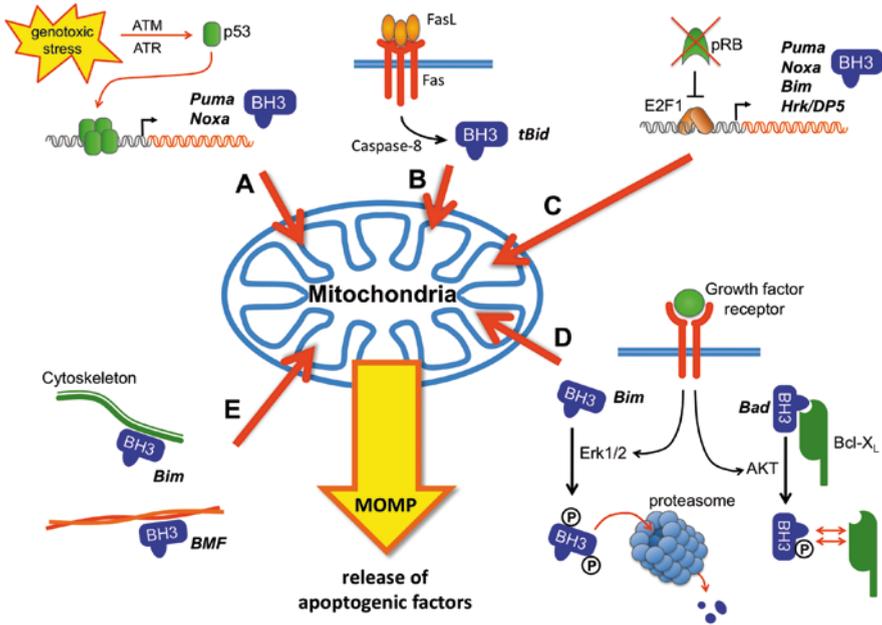


Fig. 4 Multiple death and survival pathways converge on mitochondria via BH3 only proteins. (a) Genotoxic stress leads, via ATR and ATM kinases, to activation of p53 which stimulates expression of Puma and Noxa. (b) Upon binding of its ligand FasL, trimeric Fas receptor activates caspase-8 which proteolytically generates the active truncated Bid (tBid) from Bid. (c) Disruption of pRB liberates E2F1 to stimulate expression of Puma, Noxa, Bim, and Hrk/DP5. (d) Survival signalling from growth factor receptors leads to activation of protein kinases that inactivate BH3-only proteins. AKT phosphorylates Bad which can no longer interact with antiapoptotic Bcl-2 family members. Erk1/2 phosphorylate Bim, labeling it for proteasomal degradation. (e) Bim and BMF are sequestered by DLC proteins which tether them on microtubules or actin filaments, respectively

undergo growth arrest (allowing for DNA repair) or commit suicide by apoptosis. One important element of this DNA damage response is activation of the tumor suppressor p53 which then enters the nucleus and acts as a transcription factor stimulating expression of, for example, growth arrest genes (reviewed in Yonish-Rouach (1996)). Remarkably, genes of BH3-only proteins Noxa and Puma are controlled by p53, and through the use of knockout mice have been shown to be involved in DNA-damage-induced and p53-mediated apoptosis (Michalak et al. 2008; Villunger et al. 2003). Interestingly, transcription of Noxa and PUMA genes (as well as BH3-only proteins Bim and Hrk/DP5) is also controlled by the transcription factor E2F1 which is involved in cell-cycle progression and normally held in check by the retinoblastoma tumor suppressor pRB (Hershko and Ginsberg 2004). E2F1 therefore not only upregulates cell-cycle genes when pRB is inactivated, but also sensitizes cells to apoptosis by upregulating BH3-only proteins. Thus, there is an intricate linkage among DNA damage, cell-cycle processes, and the mitochondrial apoptotic machinery involving the upregulation of BH3-only proteins.

Growth Factor Signalling Bcl-2 was first discovered as an unusual oncogene because it did not promote cell-cycle progression but rendered cells independent of growth factors normally required for survival (Vaux et al. 1988). Binding of these growth factors to their receptors triggers the activation of intracellular protein kinases, such as AKT, PKA, or MAPK. It turned out that BH3-only proteins are among the substrates of these kinases. BH3-only protein Bad was shown to be phosphorylated by AKT and/or PKA, leading to disruption of its interaction with antiapoptotic Bcl-2 family proteins (Datta et al. 1997; del Peso et al. 1997; Zhou et al. 2000) Bim can be phosphorylated by Erk1/2 at multiple sites promoting its proteasomal degradation (Ley et al. 2003). Thus growth factor receptor signaling elicits survival cues by phosphorylating and inactivating proapoptotic BH3-only proteins.

Anoikis Cell–cell contacts are crucial for cell survival; their disruption causes a special form of apoptosis that has been termed anoikis. BH3-only proteins BMF and Bim are tethered to the actin cytoskeleton or microtubules, respectively, by physical interaction with dynein light-chain proteins (Day et al. 2004). Bim was shown to become liberated from microtubules during apoptosis (Puthalakath et al. 1999). Loss of cell–cell contact was shown to unleash BMF, thus promoting anoikis (Puthalakath et al. 2001). However, in both cases the mechanism determining how loss of cell–cell contacts might unleash the BH3-only proteins from cytoskeleton has not been clarified, and it is not clear whether, for example, microtubule-poisoning drugs trigger apoptosis by promoting release of Bim.

Extrinsic Apoptotic Pathway An important pathway of apoptosis is initiated by ligation of so-called death receptors, for example, Fas or TRAIL (reviewed in Lavrik et al. 2005). Although these trigger activation of caspase-8 and/or -10, this is not always sufficient to initiate the effector caspases which in addition requires the release of apoptogens from mitochondria (Scaffidi et al. 1998). An important hub connecting the two apoptotic pathways is BH3-only protein Bid which becomes cleaved by caspase-8 to generate its active form, truncated Bid (tBid) which then initiates MOMP (Li et al. 1998; Luo et al. 1998) by binding to and inducing the oligomerization of Bax and Bak at the mitochondrial membrane (Eskes et al. 2000; Wei et al. 2000).

Collectively, mitochondria lie at the heart of several apoptotic pathways that all converge on multidomain Bcl-2 family proteins to trigger MOMP and release proapoptotic proteins. The most compelling evidence for a requirement of proapoptotic multidomain Bcl-2 family members in MOMP came from Bax/Bak-deficient mice; cells derived from these animals did not display MOMP after treatment with apoptotic stimuli, showing that Bax and Bak are essential players in MOMP. So how do these Bcl-2 family members increase MOM permeability?

Control of MOMP by Bcl-2 Family Proteins One idea for how Bcl-2 family proteins might regulate MOMP is based on early observations on the sudden increase in mitochondrial membrane permeability caused by calcium overload or reactive oxygen species (Petit et al. 1998; Scarlett and Murphy 1997). This process has been called mitochondrial permeability transition (MPT) and is initiated at the inner membrane which becomes permeable to small molecules. This results in the loss of the mitochondrial membrane potential ($\Delta\Psi$), osmotic swelling of the mitochondria and subsequent rupture of the OM which ultimately leads to

the release of cytochrome *c* from intermembrane spaces. Involvement of a large permeability transition pore complex (PTCP) has been described that contains the OM protein voltage-dependent anion channel (VDAC), the IM protein adenine nucleotide transporter (ANT), and the matrix protein cyclophilin D (see Garrido et al. (2006) for review).

There are several lines of evidence for Bcl-2 family proteins regulating MPT by interacting with PTCP components (reviewed in Kumarswamy and Chandna (2009)). However, early reports on interactions between Bcl-2 family proteins and VDAC (Shimizu et al. 1999) have subsequently been questioned (Rostovtseva et al. 2004). Moreover, biochemical and structural analyses revealed that Bax-mediated cytochrome *c* release can occur in the absence of structural and functional impairments of mitochondria (von Ahsen et al. 2000). Furthermore, through the use of knockout mice and genetic methods it was shown that Bcl-2 family-induced cell death requires neither VDACS (Baines et al. 2007) nor cyclophilin D (Baines et al. 2005; Nakagawa et al. 2005); rather cyclophilin D was involved in necrotic cell death in these models. From these latter studies it appears that MPT may play a more prominent role in necrosis than in apoptosis.

A plethora of experiments rather suggest that activated Bax and/or Bak oligomerize and form channels in the MOM whose diameter is large enough for releasing cytochrome *c* and other apoptogens (see Fig. 3) and that the subsequent MOMP occurs in the absence of MPT (Kim et al. 2000; Korsmeyer et al. 2000). In further experiments, activated recombinant Bax was able to form supramolecular membrane channels through reconstituted vesicular membranes in the absence of IM and other proteins (Kuwana et al. 2002). Also patch-clamping analyses of mitochondria from cells derived from Bax- or Bak- (or VDAC1- and VDAC3-) deficient mice revealed pore-forming ability of Bax and Bak in the absence of VDAC1, 3 (Martinez-Caballero et al. 2009). The precise structure and composition of the putative Bax/Bak pore is still unknown.

Another mechanism regarding how Bcl-2 family proteins might regulate MOMP has been deduced from their roles in mitochondrial dynamics. Mitochondrial membranes exist in an equilibrium of fusion and fission which in healthy cells is shifted towards formation of an extensive tubular network whereas in apoptotic cells fission is dominant over fusion processes (reviewed in Autret and Martin (2009)). Bax as well as Bak are interacting with the regulators of mitochondrial dynamics, Mitofusin 1 and 2, thus influencing their heterotypic interactions on the MOM and hence shifting mitochondrial dynamics in apoptotic cells towards fission (Brooks et al. 2007; Hoppins et al. 2011). It was suggested that cytochrome *c* (and other apoptogens) might be released in the course of this fission process that involves formation of so-called hemifusion intermediates which would entail the transient opening of the intermembrane space towards the cytoplasm (Montes-suit et al. 2010). However, detailed analyses revealed that cytochrome *c* release precedes mitochondrial fission (Arnoult et al. 2005). Moreover, overexpression of antiapoptotic Bcl-2 family members prevented cytochrome *c* release but not Bax/Bak-induced mitochondrial fission, suggesting that MOMP and mitochondrial fission are separable processes (Sheridan et al. 2008). Recently, nonactivated Bax has been shown to promote mitochondrial fusion and by this mechanism sensitizes cells to MPT and necrosis (Whelan et al. 2012).

The Effects of Phytochemicals on Mitochondrially-Regulated Cell Death

The toxicity of many phytochemicals has been exploited for therapy, most notably for the treatment of cancer. Cytotoxic anticancer therapy includes structurally diverse drugs that are derived from different plants, including etoposide, vinca alkaloids, and taxenes. However, none of these drugs acts directly on mitochondria nor on mitochondrial regulators of cell death. Instead they damage cells, eliciting different stress responses that ultimately converge on mitochondria, often through the regulation of different BH3-only proteins. Thus, these drugs fall into a very large group of manmade chemicals and natural products that indirectly trigger mitochondria-dependent cell death.

In contrast, there is a much smaller class of compounds that act directly on mitochondria or directly on proteins that regulate the release of apoptogens. This smaller group includes synthetic molecules (Bombrun et al. 2003; Peixoto et al. 2009) and phytochemicals such as Gossypol (Kitada et al. 2003; Zhang et al. 2003b), which is derived from the cotton plant, Purpurogallin (Kitada et al. 2003), and Chelerythrine (Chan et al. 2003), which is derived from Greater celandine.

Gossypol and Purpurogallin are polyphenols identified by screening a small library of natural products for compounds that interfere with the interaction between Bcl-2 and Bad. Chelerythrine is a benzophenanthridine alkaloid identified by screening 10^5 natural product extracts for compounds capable of displacing a Bax-derived peptide from Bcl-XL (Chan et al. 2003). Both screening approaches are designed to identify molecules that mimic endogenous BH3-only proteins such as PUMA or Noxa. Several synthetic chemicals with BH3-mimetic properties (e.g., ABT737; reviewed in Stauffer (2007)) have also been identified. However, Gossypol can also kill cells by reactive oxygen species-dependent mechanisms (Barba-Barajas et al. 2009) and Bax/Bak null cells are not protected from the effects of Gossypol (Vogler et al. 2009). Similarly, Chelerythrine also kills Bax/Bak null cells (Wan et al. 2008). Moreover, the ultrastructural changes in cells dying after treatment with Gossypol, Chelerythrine, or synthetic BH3-mimetics are all different (Vogler et al. 2009). Together these data suggest that although Gossypol and Chelerythrine may kill via direct action on Bcl-2 proteins, this is not the only way they kill cells. Although Gossypol shows anticancer activity in xenograft models and has been tested in patients with advanced malignant disease (Flack et al. 1993; Stein et al. 1992), its therapeutic usefulness is limited by its side effects (Wu 1989), perhaps associated with Bax/Bak-independent death processes. Consequently a modified form of Gossypol, Agossypol, has been made with the aim of reducing the adverse effects associated with treatment (Becattini et al. 2004). Agossypol acts as a BH3-mimetic and kills cancer cells *in vitro*, however, whether it has *in vivo* activity against human malignancies and fewer adverse effects than Gossypol has not been established.

Disease and Mitochondrially-Regulated Cell Death

Cell death regulated by mitochondria has been linked to a wide range of disease states. Consequently the idea that small molecules able to interfere with mitochondrially regulated cell death will be potential therapeutics has been discussed and researched over many years and by many scientists. As discussed in this chapter, mitochondrial cell death pathways are regulated at several levels:

1. Initiation/signaling to mitochondria, most notably BH3-only proteins
2. Pore opening mechanisms (Bax/Bak; PTCP components)
3. Death mechanisms elicited by the released apoptogens

In principle, each of these levels might be targeted by small molecules.

It is clear that a wide range of anticancer drugs activates the intrinsic apoptotic pathway, triggers the release of apoptogens, and thus kills cancer cells. Typically, these act by regulating the expression or activity of BH3-only proteins, and many currently used cancer chemotherapeutics that act in this way are derived from phytochemicals (e.g., Etoposide). Gossypol acts directly on the mitochondria, and although it may not ever be used in the clinic, other BH3 mimetics that act through a similar mechanism will be. Small molecules that mimic Smac are also in clinical development for treating cancer, although none of these are phytochemicals (Fulda and Vucic 2012). Thus, small molecules targeting each of the key steps in mitochondrially regulated cell death have been identified and some are clinically useful.

Mitochondrially controlled cell death has also been implicated in several other pathologies including ischemic injury, and neurodegenerative diseases such as Parkinson's disease (Tatton et al. 2003), Huntington's disease (Vis et al. 2005), and Alzheimer's disease (Kudo et al. 2012). In these cases (and in contrast to cancer therapy) the goal is discovery of small molecules that block cell death, and blocking mitochondrially regulated cell death is indeed possible in experimental models of at least one degenerative disease (Kudo et al. 2012). However, it is not clear that preventing cell death is equivalent to preserving cell function. Until this point is resolved, searching for small molecules that block mitochondrially regulated cell death may be futile as blocking cell death need not be associated with any improvement in patients with degenerative diseases. Thus, at the current time, the search for compounds that either directly kill cancer cells by selectively activating the mitochondrial cell death pathway or sensitize cancer cells to mitochondrial cell death pathways induced by existing anticancer drugs is more likely to be productive.

Concluding Remarks In this chapter we have introduced the death-inducing proteins released from mitochondria and described what is known of the mechanisms that govern their release. In addition, we have briefly discussed some phytochemicals that affect these processes and considered this information in context of human disease. It is clear that phytochemicals can trigger the release of mitochondrial proteins and thus induce cell death in several different ways and that this can be exploited for

therapeutic gain, at least in treating cancer. Identifying phytochemicals that are able to mimic the effect of apoptogens (e.g., phytochemicals that are Smac-mimetics) may only require the application of the appropriate high-throughput assays.

HtrA2 substrates play a range of roles in diverse biological processes. The normal roles of KIAA0251 and KIAA1967 are not yet clear. The functional significance of cleavage by HtrA2 has not been demonstrated for all substrates.

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Cell-Death—Inducing Mechanisms of Cancer Chemopreventive Agents

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Abstract Our increasing knowledge about health and disease suggests that vegetarian diets rich in fruits and vegetables have a significant impact in prevention and therapy of multiple types of cancer. Phytochemicals from both dietary and nondietary origins inhibit cancer growth and progression of human cancers by interacting at the cellular and molecular levels. The mitochondrion is the key organelle that provides energy to cells for their growth and survival. In addition, mitochondria play important role in cancer cell apoptosis and may also regulate autophagy. Cancer cells have high proliferative and less apoptotic activities; therefore, agents that induce apoptosis selectively in cancer cells are desired for the treatment of cancer. Some dietary phytochemicals target mitochondria causing destruction of mitochondrial membranes leading to the discharge of proapoptotic mitochondrial proteins, which initiate apoptotic cell death in cancer. Although the role of autophagy is debated as a mechanism of cell death or cell survival, many phytochemicals modulate autophagy in cancer cells. Therefore, selective targeting of cell-death pathways by phytochemicals in cancer cells could be an attractive strategy for the prevention and treatment of cancer.

Keywords Cancer chemopreventive agents • Phytochemicals • Mitochondria • Apoptosis • Autophagy

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Introduction

Living organisms are broadly classified as either unicellular or multicellular organisms. The complexity of many different cell types coming together in multicellular organisms mandates stringently regulated processes for cell division, proliferation, and differentiation, which are controlled by many signaling pathways. The rate of cell division is controlled by the process of apoptosis and differentiation so that the organism is not overburdened with unwanted cells. In humans, around 10^7 cells die in the body every second (Reed 2010) and a variety of pathways are used to coordinate cell death during development and morphogenesis to control cell numbers thus eliminating damaged cells. By this process, the body ensures that only healthy cells survive in the body and cells having any mutation or change that might be harmful, are eliminated. But some cells escape from the usual barricade mechanisms within the body and they further divide, accumulate, and may turn cancerous. These cells acquire the property of indefinite cell division and resist programmed cell death or apoptosis. Defects or resistance to normal programmed cell-death mechanisms are prerequisites in the pathogenesis of tumors.

Cell death as a process becomes very important in maintaining homeostasis and helps to evade pathogenesis. There are three mechanisms of cell death namely apoptosis, autophagy, and necrosis (Ouyang et al. 2012). Other than these mechanisms, anoikis is a special case of apoptosis in response to inappropriate cell/extracellular matrix (ECM) interactions, leading to complete detachment and eventually death. The importance of anoikis *in vivo* can readily be seen when alterations that perturb its normal control enhance tumor metastasis, a process that requires cells to survive in totally inappropriate ECM environments. Cancer cells are known to develop resistance towards these cell-death mechanisms and thereby continue to divide and proliferate in spite of accumulating mutations and other defects. Agents triggering cell death in cancer cells or sensitizing them to normal cell-death mechanisms are used as cancer therapeutics (Reed 2006). Natural plants and vegetation have been well studied and documented by ancient literature for their medicinal values. With further development of technology, there have been studies on identifying active principles from these plants or various constituents of our daily diet and identifying their medicinal activities including anticancer efficacies. These studies have resulted in identification of numerous compounds or molecules from plant origins with anticancer activity against various human cancers including skin cancer, prostate cancer, oral cancer, and breast cancer (Surh 2003). Although there are several factors that affect tumor cells, among them the most important is the daily lifestyle or diet. In this chapter, we review the three major death mechanisms and try to understand how the natural or chemopreventive agents modulate these processes and hence function as anticancer agents in cancer cells.

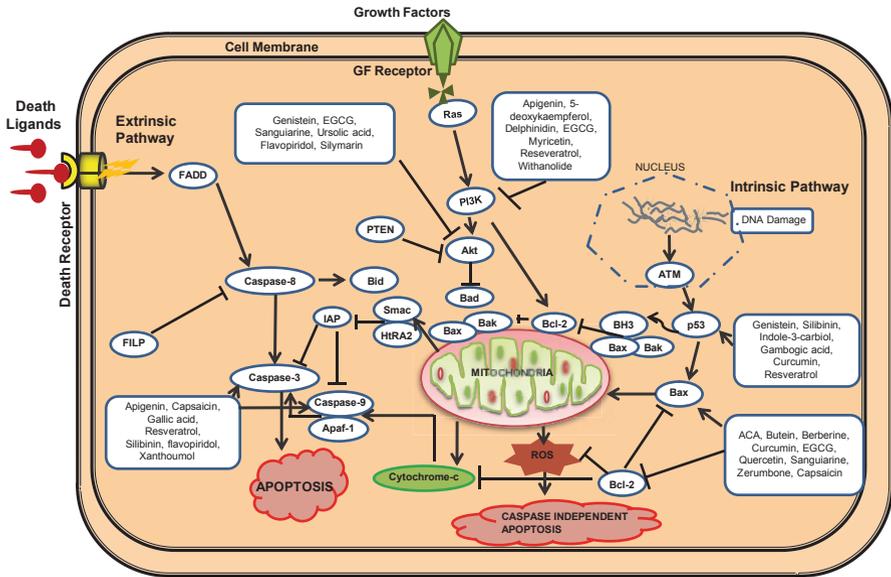


Fig. 1 Diagram depicts various death-inducing pathways and potential modulatory targets for chemopreventive agents in cancer cells. Phytochemicals act as modulators of apoptosis pathways and also trigger the expression of proteins that play a central role in apoptosis. For example, apigenin, capsaicin, gallic acid, resveratrol, silibinin, and flavopiridol activate caspase-3 and -9 leading to apoptosis. However, ACA, butein, berberine, curcumin, EGCG, quercetin, sanguinarine, zerubone, and capsaicin downregulate Bcl-2 and upregulate Bax which plays a major role in the intrinsic pathway of apoptosis induction

Phytochemicals in Chemoprevention of Cancer

Plants have been the main source of a significant percentage of potent anticancer agents that are discovered, developed, and being used in clinical practice. Prevention of cancer by natural compounds needs better understanding of the mechanistic actions including their effect on cancer cell proliferation, angiogenesis, invasion, migration, and metastasis. After almost three and a half decades of research following the coining of the term “chemoprevention” by Michael Sporn in 1976, it is now getting recognition as a potent strategy against cancer prevention and control (Bode and Dong 2009). Chemoprevention aims to arrest the cancer in its early stages so that disease eventually does not lead to the invasive and metastatic stage and seeks to reverse the preneoplastic condition. Phytochemicals have been isolated and characterized from the different sources of plants including fruits, vegetables, spices, beverages, cereals, and many others. Figure 1 shows various dietary agents from fruits, vegetables, dietary supplements, and medicinal spices that inhibit different processes of cancer including cell invasion, migration, metastasis, and angiogenesis, along with attenuating the exponential growth of cancer. Although a small portion of

natural compounds have been explored, a large chunk of marine life and other potent sources are open for development and discovery of new drugs or chemopreventive agents. Thus phytochemicals serve as excellent molecules to inhibit the promotion and progression of carcinogenesis, to remove genetically damaged, preinitiated, or neoplastic cells from the body by inducing apoptosis or cell cycle arrest.

The incidence of cancer is increasing worldwide, however, fruit and vegetable intake has been shown to reduce the risk of cancer in numerous epidemiological studies (Block et al. 1992). Recently, biochemical and molecular approaches have been applied to select naturally occurring dietary substances that showed more control over the key molecules regulating cancer pathways. These biomolecules are potentially able to modulate intracellular signaling pathways and may provide a molecular basis of chemoprevention. Table 1, shows various phytochemicals including silibinin, resveratrol, epigallocatechin-3-gallate (EGCG), fisetin, capsaicin, curcumin, and genistein which have been reported to cause cell death via both extrinsic and intrinsic pathways of apoptosis in cancer cells. These phytochemicals have been explored in different types of cancer such as prostate, lung, breast, cervical, oral, and colon cancer at different doses, and it has been estimated that dietary modification may prevent more than two-thirds of human cancer (Surh 2003). These agents have also been explored for their role as chemosensitizing and radiosensitizing agents, wherein they function by countering the resistance mechanisms in cancer cells (Garg et al. 2005; Nambiar et al. 2011). The defective apoptotic pathways are well-known causes of carcinogenesis and, in addition, are one of the resistance mechanisms acquired by cancer cells in response to various therapeutic agents. However, cancer cells acquire resistance to apoptosis by overexpression of antiapoptotic proteins and/or by the downregulation or mutation of proapoptotic proteins. Therefore, identification and exploration of pathways that are involved in carcinogenesis could facilitate the use of dietary constituents as a key strategy to prevent cancer development. As shown in Fig. 1, phytochemicals modulate extrinsic and intrinsic pathways at the molecular level and also target the significant proteins that constitute the apoptosis core machinery including Bcl-2, Bax, cytochrome-c, and caspases which mediate apoptosis.

Death of Cancer Cells: Role of Mitochondria

All cell organelles in the mammalian cells serve a unique and well-demarcated purpose. Among these organelles, one that draws rigorous attention is the aptly called “powerhouse of the cell,” the mitochondrion. Cellular energy is almost solely controlled by the mitochondrion, which ultimately produces the energy for cell. As much as it governs life, it also governs one of the most illustrious mechanisms of cell death, apoptosis. Mitochondrial involvement in various diseases is also very well documented. The role of mitochondria in pathogenesis gained slow and steady importance in the previous two decades of research and is now a well-recognized target even for cancer therapeutics (Tatarkova et al. 2012). Mitochondrial membrane permeabilization is one of the central steps to apoptosis. The aim of major

Table 1 Molecular targets of cell death of cancer chemopreventive phytochemicals

Phytochemicals	Source	Molecular Targets of Cell Death	References
Curcumin	Turmeric (<i>Curcuma longa</i>)	Induction of apoptosis <i>via</i> downregulation of Bcl-2 and Bax upregulation; activation of caspase-3 and 9 Induces autophagy by inhibiting autophagosome formation; enhances Beclin 1 and LC3-II levels	Guo et al. 2012; Kunwar et al. 2012, Aoki et al. 2007; Jia et al. 2009
Silibinin	Milk thistle plant (<i>Silybum marianum</i>)	Induces apoptosis <i>via</i> cleavage of PARP, activation of caspase-9 and 3, induction of intracellular Ca ⁺² levels Induces autophagy by enhancing Beclin 1 and LC3 II levels and generation of ROS	Ramasamy and Agarwal 2008; Kim et al. 2009; Duan et al. 2010
EGCG	Green tea (<i>Camellia sinensis</i>)	Apoptosis induction by increased expression of Bax and decreased expression of Bcl-2, Bcl-xL ; enhanced Fas ligand expression; decreased survivin expression; increased intracellular Ca ⁺² levels	Kuo et al. 2003; Onoda et al. 2011; Wu et al. 2009
Fisetin	Smoke tree (<i>Cotinus coggygria</i>)	Causes apoptosis through DR-3 suppression and NF-κB activation; caspase-7, 8, 9 and PARP cleavage Induces autophagy by inhibition of mTOR complexes	Szliszka et al. 2011; Yang et al. 2012; Suh et al. 2010
Grape Seed Extract (GSE)	Grapes (<i>Vitis vinifera</i>)	Activates both intrinsic and extrinsic apoptotic pathways; reduces FAK levels; enhances ROS production	Kaur et al. 2006; Derry et al. 2012
Garcinol	Kokum (<i>Garcinia indica</i>)	Causes apoptosis through downregulation of NF-κB	Ahmad et al. 2010
Genistein	Soybeans (<i>Glycine max</i>)	Induces apoptosis <i>via</i> phosphorylation and activation of p53 and decrease of ratios of Bcl-2/Bax and Bcl-xL/Bax	Lian et al. 1999; Pavese et al. 2010
Resveratrol	Red grapes (<i>Vitis vinifera</i>)	Induces apoptosis by targeting Bax, Bak, Bcl-2, and downregulates survivin	Athar et al. 2009
Sanguinarine	Bloodroot (<i>Sanguinaria canadensis</i>)	Induces apoptosis through up-regulation of Bax, Bak, Bid and down-regulation of Bcl-2 and Caspase-3 activation.	Malikova et al., 2006
Xanthohumol	Hop (<i>Humulus lupulus</i>)	Induces apoptosis through up-regulation/activation of caspase-3, 8 and 9 and down-regulation of Bcl-2 expression	Pan et al., 2005

Table 1 (continued)

Phytochemicals	Source	Molecular Targets of Cell Death	References
Flavopiridol	Rosewood (<i>Dysoxylum binectariferum</i>)	Induces apoptosis <i>via</i> activation of the Bid, cytochrome <i>c</i> , caspase-9 and 3	Achenbach et al., 2000
Capsaicin	Chili pepper (<i>Capsicum</i>)	Down-regulates expression of Bcl-2, Bcl-xL and survivin	Bhutani et al., 2007
Acetoxychavicol acetate (ACA)	Blue ginger (<i>Alpina galangal</i>)	Suppresses TNF-induces NF- κ B-dependent expression of survivin and Bcl-2	Ichikawa et al., 2005
Anacardic Acid	Cashew (<i>Anacardium occidentale</i>)	Inhibits Bcl-2, Bcl-xL, cFLIP, cIAP-1 and survivin	Sung et al., 2008
Berberine	Barberry (<i>Berberis vulgaris</i>)	Induces apoptosis by induction of mitochondrial membrane potential, reduces Bcl-2 and Bcl-xL levels; increases Bax, Bak, caspase-3 activation, release of cytochrome <i>c</i> , and cleavage of PARP	Katiyar et al., 2009
Butein	Chinese Lacquer Tree (<i>Toxicodendron vernicifluum</i>)	Down-regulates the expression of NF- κ B-regulated gene products such as IAP-2, Bcl-2 and Bcl-xL	Pandey et al., 2007
Capsaicin	Chili pepper (<i>Capsicum</i>)	Down regulates expression of Bcl-2, Bcl-xL and survivin	Bhutani et al., 2007
Gambogic acid	Gamboge tree (<i>Garcinia hanburyi</i>)	Prompts apoptosis through up regulation p53 and down regulation of Bcl-2	Rong et al., 2009
Indole-3-carbinol	Broccoli (<i>Brassica oleracea</i>)	Induces apoptosis through activation of Bax, PARP cleavage and down regulation of Bcl-xL, Bcl-2, BAD	Aggarwal and Haruyo 2005
Plumbagin	Plumbago (<i>Plumbago europaea</i>)	Inactivates NF- κ B and downregulates Bcl-2	Ahmad et al., 2008
Allicin	Garlic (<i>Allium sativum</i>)	Induces cytochrome-c release, and enhances cleavage of caspase-9 and 3, and Fas mediated apoptosis	Bat-Chen et al., 2010; Zhang et al., 2010
Apigenin	Parsley (<i>Petroselinum crispum</i>)	TNF- α , NF- κ B, modulates Bax and Bcl-2 levels, induces cytochrome <i>c</i> release, PARP cleavage	Shukla and Gupta, 2010
Delphinidin	Cranberries (<i>Vaccinium oxycoccus</i>)	Induces apoptosis by induction of Bax and inhibition of Bcl-2 protein expression; increases cleaved caspases-3 and 9 in prostate cancer cells	Hafeez et al., 2008
Gallic acid	Pomegranate (<i>Punica granatum</i>)	Induces apoptosis by induction of cleavage of caspase-4, 3, 9	Hsu et al., 2011
Gingerol	Ginger (<i>Zingiber officinale</i>)	Inhibition of Bcl-2 expression and induction of Bax; induction of cytochrome <i>c</i> release	Nigam et al., 2009

Table 1 (continued)

Phytochemicals	Source	Molecular Targets of Cell Death	References
Withanolide	Ashwagandha (<i>Withania somnifera</i>)	Induces apoptosis by induction of Bax and inhibition of Bcl-2 levels; increases cleaved caspases-3 and 9	Zhang et al., 2011
Zerumbone	Wild ginger, (<i>Zingiber zerumbet</i>)	Induces apoptosis by induction of Bax and inhibition of Bcl-2 levels	Kim et al., 2009

chemotherapeutic agents has been the induction of apoptosis in cancer cells. Hence, targeting molecules controlling the mitochondrial membrane permeability is one of the most common mechanisms of cell death induction in cancer cells. Second, targeting the cellular energetic pathways could also be a triggering factor for cell death induction. Another recent study suggests that manipulating and targeting the number of mitochondria could also be one of the ways of targeting cancer cells. It is now known that the mitochondrial number per cell is regulated by two processes of fusion and fission, fission leading to doubling the number and fusion leading to reducing the number to half. In the study by Rehman et al. (2012) it was shown that by tipping the balance more towards fusion rather than fission, they could dramatically reduce cell proliferation and growth of lung cancer cells in mice. Even though novel ways of mitochondrial targeting are being explored, the most recognized involvement of mitochondria remains on cell death induction via apoptosis.

Although several years of research and observations have explained the process and morphology of apoptotic cells, such as chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and cell shrinkage (Ferreira et al. 2002), with the recent advancement in technology we are capable of understanding the process of cell death at the cellular as well as molecular level. It is now not only possible to identify and decipher the extent of involvement of cell organelles, but also of the smallest of molecules coupled with the process. Hence, each and every molecule regulating mitochondrial function could be explored as a target for cancer prevention and control.

Mitochondrial Pathways of Apoptosis: Potential Targets of Phytochemicals

Mitochondrial involvement in the cell death program proceeds through two main pathways namely, intrinsic and extrinsic pathways. The mitochondrial pathway of apoptosis functions in response to various types of intracellular stress including growth factor withdrawal, DNA damage, unfolding stresses in the endoplasmic reticulum, and death receptor stimulation (Khosravi-Far and Esposti 2004). The extrinsic apoptotic pathway is activated through the death receptors such as tumor necrosis factor (TNF) receptor, TNF-related apoptosis-inducing ligand (TRAIL)

receptor, and APO1 receptor (also called Fas or CD95). Death receptors are cell surface receptors which are integral cell membrane proteins that transmit apoptotic signals initiated by specific ligands such as Fas ligand, TNF- α , and TRAIL. Although there are differences in the signaling pathways stimulated by the different death receptors, they perform a central role in inducing apoptosis and can activate the downstream cascade after the binding of its specific ligand. The ligand binding causes a conformational alteration in the intracellular domains of the receptors, which exposes the death domain and allows the recruitment of several apoptotic proteins to the receptor forming protein complexes. These protein complexes are known as death inducing signaling complexes (DISC). The establishment of DISC initiates the caspase cascades and therefore the stimulation of apoptosis via this mechanism is very quick (Khosravi-Far and Esposti 2004). Binding of TNF- α to TNFR1 results in receptor trimerization and clustering of intracellular death domain, that is, TNFR-associated death domain (TRADD) which associates with FADD and ultimately leads to the initiation of apoptosis via the recruitment and cleavage of procaspase-8. Similarly, in Fas receptor activated apoptosis, the process is triggered by the Fas and CD95 ligands, which leads to binding of TRAIL to the DR4 or DR5 receptor and activates the TRAIL death-receptor apoptosis pathway (Guicciardi and Gores 2009). Following the reception of stress signals, proapoptotic Bcl-2 family proteins are activated and subsequently interact with and inactivate antiapoptotic Bcl-2 proteins.

The Bcl-2 is a family of proteins of pro- and antiapoptotic function. On the basis of structural homology and respective functions, Bcl-2 proteins had been classified into three groups. The first group shows the antiapoptotic function with structural homology in BH domains (BH1–BH4), for example, Bcl-2, Bcl-xL, and Mcl-1; the second group includes the pro-apoptotic members with three BH domains, for example, Bax and Bak; and the third group of proapoptotic proteins shows homology within the BH3 domain known as Bad, Bid, or Bim (Garrido et al. 2006). These proteins potentially participate in signaling, which changes the mitochondrial membrane dynamics. On receiving an apoptotic signal, the proapoptotic members enhance the mitochondrial membrane permeability and hence lead to release of cytochrome *c*, followed by apoptosome complex formation. The level of Bax and Bcl-2 ratio is a pivotal factor and plays a significant role in determining future survival or apoptotic death of the cell. After the apoptosome formation, the program cell death is triggered and is performed by a family of highly conserved cysteinyl aspartate-specific protease known as caspases. Caspases cleave a large number of cellular substrates and thus dismantle the cell. Caspases-8 and -10 are activated by the death receptor of cell-like Fas/CD95, TNFR, DR-3, and DR4/5. caspase-9 is activated through the cytochrome *c* release of mitochondria, and caspases-8, -10, -9 are considered as most upstream caspases in apoptotic signaling (Fan et al. 2005). Caspases-3, -6, and -7 are the downstream caspases activated by upstream caspases or the signal cascade of the death receptor of the cell. Most of the cancer cells acquire resistance to apoptotic stimuli via overexpression of antiapoptotic proteins such as Bcl-2 or under expression of proapoptotic factors. Many phytochemicals have the potential to modulate the levels of these regulatory proteins, and thereby,

lead to caspase activation and apoptosis. Survivin is an inhibitor of apoptosis protein (IAP), which is highly expressed in cancer cells and is a target for chemopreventive agents. It has been observed that its expression interferes with caspase-9 and prevents caspase activation, and as a result inhibits apoptosis (Altieri 2006). It is reported that mitochondrial expression of survivin is not found in normal cells, whereas mitochondrial survivin is absolutely related with tumor transformation of cells (Altieri 2003, Dohi et al. 2004). Survivin increases apoptotic resistance in cancer cells mainly by blocking mitochondrial intrinsic pathways.

Autophagy as a Target of Phytochemicals for Cancer Prevention

Autophagy, as the term itself suggests is a cellular degradation mechanism in response to cellular stress. Over the past decade, many studies have established the exact mechanism of autophagic process, ranging from how it is executed, to the protein machinery involved, but until now, the context in which autophagy is triggered is still unclear. Questions remain about its utility in the cell. Is it just a homeostasis-maintaining cytoprotective mechanism or could it be a cytotoxic mechanism in a defined set of conditions or in a disease state? Most studies advocate that autophagy is a cytoprotective mechanism providing the cells support to sustain survival, but then it becomes tricky to explain why so many cytotoxic agents induce autophagy. If it is indeed a prosurvival mechanism, then it remains ambiguous why genes involved in autophagy are frequently mutated in human cancers (Mathew et al. 2007). Different explanations have been provided for this process; one plausible explanation for this paradoxical process could be that autophagy in limited condition is prosurvival but if it exceeds the rate of cellular synthesis, then it may promote cell death. In the case of apoptosis-resistant tumors, autophagy helps to survive metabolic stress, hence helping those tumor cells to flourish and therefore autophagic inhibition could be seen as a mechanism to sensitize apoptosis-resistance cells to metabolic stress-induced death.

As a target in cancer prevention, autophagy play a vital role as it can inhibit tumor initiation by removing the damaged cells and limiting genomic instability. Furthermore, autophagy may also be able to limit chronic inflammation, which is considered to be a known precursor to carcinogenesis. This notion is well supported by the fact that autophagy-deficient mice are comparatively more tumor prone (Takamura et al. 2011). Also, use of autophagic inducers such as rapamycin and metformin reduce the tumor initiation process. Autophagy is regulated through the PI3K/AKT/mTOR pathway, Beclin 1 complex, Bcr-Abl, and FOXO signaling (Sandri 2012; Wang et al. 2011). The PI3K signaling network plays critical role in the regulation of cell growth, survival, proliferation, differentiation, and motility, but when dysregulated can lead to oncogenic transformation. PI3K pathway activates AKT and eventually mTORC1, which is one of its direct targets. mTORC1 is needed for protein translation and supports net cell-mass accumulation by inhibiting autophagy (Jung et al. 2010). The inhibitory mechanism of mTORC1 is brought about by inhibitory phos-

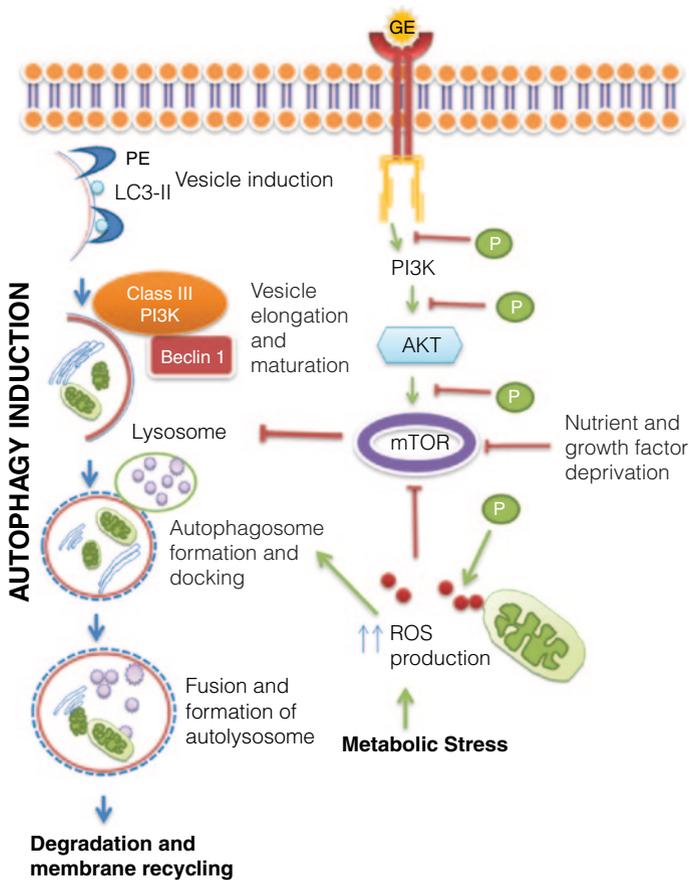


Fig. 2 The effect of phytochemicals on autophagic death in cancer cells. Autophagy involves (1) vesicle induction (2) vesicle elongation and maturation (3) autophagosome formation and docking (4) fusion and formation of autolysosome which is regulated by PI3K/Akt/mTOR, RTK signaling, ROS and nutrient availability. (P) depicts the modulation of autophagy by various phytochemicals; PE, phosphoethanolamine

phorylation of ULK1 and ATG13 proteins that are involved in phagophore initiation complex formation, especially when there is an abundance of nutrients and growth factors. When nutrients are limited, it dissociates from the ULK1 complex, initiating the autophagy process. The vesicle nucleation and assembly of the phagophore requires a Class III phosphatidylinositol 3-kinase (PI3K-III) complex containing the proteins VPS34, p150, ATG14, and Beclin 1. It is followed by the phagophore elongation which requires Atg16 and LC-3. The phagophore then elongates to form the autophagosome, which encapsulates cytoplasmic material and fusion with lysosome that involves proteins including Rab7, SKD1, LAMP1 and LAMP2, eventually delivering its cargo to the lysosome. These proteins that are involved in the formation of autophagosome and upstream signaling pathways governing the process could be

potential targets of cancer chemopreventive agents. Autophagy as a process helps in the removal of unwanted elements from the cytoplasm and suppresses the accumulation of toxic protein aggregates and the production of damage-inducing reactive oxygen species (ROS). The sustained state of oxidative stress is also a prelude to the genome and tissue damage, inflammation, and cell death.

Autophagy is also linked to mitochondria-mediated apoptosis. For instance, the role of autophagy in controlling the number of damaged mitochondria (mitophagy), which are a major source of ROS production in mammalian cells, is not yet well understood. Hence, both autophagy inducers and inhibitors are used in cancer therapeutics. Several of the natural compounds of plant origin, such as curcumin, silibinin, resveratrol, paclitaxel, quercetin, genistein are shown to induce these autophagic pathways as potential therapeutic agents to deal with carcinogenesis (Fig. 2). The context dependent role of autophagy and how it could be used in cancer therapeutics is very well summarised in the review by Cheong et. al. (Cheong et al. 2012). The successful targeting of autophagy in cancer therapy by natural compounds would require molecular analysis of the components of distinct forms of autophagy, identifying the context of operation with respect to the tumor stage as well correlating how this process is related to apoptotic mechanisms in cancer cell.

Phytochemicals Inducing Cell Death in Cancer Cells

Curcumin

Curcumin, is isolated from *Curcuma longa* and has been extensively evaluated for its anti-carcinogenic and chemopreventive efficacies in a wide range of cancers. Its potential anti-carcinogenic effects are exerted *via* mechanisms such as induction of cell cycle arrest, activation of tumor suppressor genes including p53 and various transcription factors such as Nrf2 and NF- κ B modulation of inflammatory signaling cascades and by inducing apoptosis (Zhou et al. 2011). These effects in turn are all known to be exerted *via* its ability to modify or alter the response of various signaling pathways including mitogenic and inflammatory responses, cell cycle regulation, and cell death regulating pathways.

Curcumin has been shown to induce both mitochondria-dependent as well as -independent cell death mechanisms. Guo et al. showed that curcumin induced apoptosis in human colon carcinoma LoVo cells was mediated *via* mitochondrial death pathway involving activation of caspase 3 and 9. Curcumin also down-regulated survivin levels in these cells (Guo et al. 2012). It has also been reported that curcumin induced cell death in nasopharyngeal carcinoma cells by inducing cytochrome c release and activation of caspase 9 and 3 (Kuo et al. 2011). Similar results were reported with dimethoxycurcumin on MCF-7 breast cancer cells, wherein curcumin could induce loss of mitochondrial potential in these cells leading to apoptosis. In another study, curcumin could alter the cellular energy status of the cells by decreasing the ATP/ADP ratio. This effect was in turn brought in by the

suppression of α , β , γ , ϵ subunits of ATP synthase. The study further showed that curcumin could reduce the levels of Bcl-2 and increase Bax levels (Kunwar et al. 2012). Curcumin was also shown to induce apoptosis *via* intrinsic pathway which was evident by increase in mitochondrial calcium accumulation in murine mammary gland adenocarcinoma. The mechanism was further confirmed by use of a mitochondrial uniporter inhibitor prior to the treatment of the cells by curcumin, which resulted in inhibition of curcumin mediated loss of mitochondrial membrane potential and eventually decreased cell death (Ibrahim et al. 2011). Recently, it has been shown that Curcumin induced cell death required Apaf-1, as Apaf-1 deficiency in these cells resulted in inhibition of caspase 3 activation by Curcumin (Gogada et al. 2011). Role of curcumin in inducing the intracellular calcium levels as a mechanism of inducing cell death was also confirmed by Wang et al., who demonstrated that curcumin induced cell death in hepatocellular carcinoma cells could be abrogated by the use of intracellular calcium chelators (Wang et al. 2012).

Another form of cell death mechanism shown by curcumin is induction of autophagy. After treatment with curcumin, levels of LC3-II and Beclin 1 have been detected to be elevated in K562 cells, suggesting that curcumin may affect autophagosome formation (Jia et al. 2009). In glioma cells, curcumin has been found to inhibit the AKT/p70S6 kinase pathway while activate ERK1/2, resulting in autophagic induction, whereas PI3K activity was not affected (Aoki et al. 2007). It is also suggested that in oral squamous carcinoma cells, curcumin induces formation of autophagic vesicles, which was confirmed by the use of an autophagic inhibitor, which suppressed the effects of curcumin on the cells. This study also showed that curcumin induced production of ROS in these cells and use of antioxidant N-acetyl cysteine (NAC) led to suppression of not only ROS but also the formation of autophagic vesicles and vacuoles, suggesting that curcumin mediated autophagy in these cells could be *via* generation of ROS and oxidative stress induction (Kim et al. 2012).

Silibinin

Silibinin is a polyphenolic flavonoid isolated mainly from the seeds of milk thistle (*Silybum marianum* (L.) Gaertn). Over a decade, many studies have shown that silibinin targets signaling pathways that are constitutively activated in cancer cells and are involved in tumor cell survival, growth, invasion and metastasis. Silibinin mediates these pleiotropic effects by targeting various signaling pathways including EGFR, IGF-1R and NF- κ B pathways in (Singh and Agarwal 2006). Silibinin induced inhibition of tumor cell growth is brought about by induction of cell cycle arrest, mainly in the G1 phase, which leads to prolonged doubling time of these cells. The induction of cell cycle arrest *via* silibinin in various *in vitro* and *in vivo* models has been attributed to its ability to reduce the levels of CDKs and cyclins and also by augmenting the levels of cell cycle inhibitory proteins, the CDK inhibitors, including Cip1/p21 and Kip1/p27. Triggering the process of cell death by silibinin is also one of its well studied mechanisms of tumor inhibition. Silibinin has been

shown to induce apoptosis in a wide range of cancer cells. Here, we summarize few of the studies depicting its role in cancer cell death.

One of the earliest reports showing the role of silibinin in cell death came in 1999 for cervical carcinoma cells (Bhatia et al. 1999), and later it was shown that silibinin inhibits both constitutive and TNF- α induced NF- κ B activation in human prostate carcinoma DU145 cells, and significantly sensitizes them to TNF- α induced apoptotic death (Dhanalakshmi et al. 2002). Further, it was also shown that silibinin could synergize with doxorubicin to enhance apoptosis induction in prostate cancer cells (Tyagi et al. 2002). The combination of silibinin was further tried along with other chemotherapeutic agents including cisplatin and carboplatin, wherein it sensitized the cells to drug induced apoptosis in human prostate cancer cells (Dhanalakshmi et al. 2003). The results obtained *in vitro* was further validated by *in vivo* models of PCa using athymic nude mice xenograft showing that the inhibition of tumor growth in treated mice were due to, in part, by silibinin induced apoptosis as evidenced by increased levels of cleaved caspase-3 (Singh et al. 2003). Silibinin was shown to down-regulate survivin which was associated with a very strong and prominent caspase-9 and -3 activation as well as PARP cleavage in bladder cancer cells (Tyagi et al. 2003). The role of silibinin in inducing cell death in bladder carcinoma cells has been further evaluated. Silibinin enhanced p53 activation which was mediated *via* ATM-Chk2 pathway, which in turn induced caspase-2-mediated apoptosis. Further, silibinin caused a rapid translocation of p53 and Bid into mitochondria leading to increased permeabilization of mitochondrial membrane and cytochrome c release into the cytosol. The study deciphered a novel mechanism for apoptosis induction by silibinin involving p53-caspase-2 activation and caspase-mediated cleavage of Cip1/p21 (Tyagi et al. 2006). Recently, it was shown that in hepatocellular carcinoma cells, silibinin activated extrinsic apoptotic pathway by up-regulating the TRAIL and DR5 and caspase-3 and -8 activation (Bousserouel et al. 2012).

Another mechanism of silibinin mediated cell death was observed in glioma cells. Kim et al. showed that silibinin could inhibit glioma cell proliferation *via* Ca²⁺/ROS/MAPK dependent mechanism *in vitro* and *in vivo*. Silibinin was shown to induce intracellular calcium levels and augment levels of ROS thereby inducing death. EGTA, calpain inhibitor or NAC could abrogate silibinin induced cell death (Kim et al. 2009). A follow-up study by Jeong et al. using calpain inhibitors showed that calpains which are cytosolic calcium activated cysteine proteases, play an important role in silibinin-induced cell death. Further analysis revealed that use of calpain inhibitor eradicates silibinin induced AIF nuclear translocation and eventually silibinin induced cell death (Jeong et al. 2011).

Apart from induction of apoptosis, silibinin is known to induce autophagic cell death mechanisms. Silibinin-induced autophagy led to increase in the conversion of LC3 I to LC3 II and an up-regulation of Beclin 1 expression, which was concomitant with p53 suppression and NF- κ B activation in human melanoma cells (Jiang et al. 2011). Use of NF- κ B inhibitor led to the inhibition of silibinin-induced autophagy, and autophagic specific inhibitor 3-methyladenine (3-MA) treatment reversed silibinin-induced p53 expression as well as NF- κ B activation. This study

suggests a positive feedback loop between p53 inhibition-mediated NF- κ B activation and autophagy (Jiang et al. 2011). Similar studies were also reported in fibroblast HT1080 cells where silibinin induced Beclin 1 expression and generated ROS, and use of NAC abrogated silibinin induced autophagy in these cells (Duan et al. 2010). Later, the same group further elucidated the mechanism of silibinin-induced autophagic cell death suggesting that autophagy induction was mediated *via* activation of p53-ROS-p38 and JNK pathways (Duan et al. 2011). In cervical cancer cells, silibinin induced autophagy as well as apoptosis *via* induction of ROS generation. The study suggested the involvement of p53-mediated ROS generation as well as ROS-mediated p53 activation. Silibinin was also found to activate JNK, which in turn induces ROS formation and therefore, silibinin might activate p53-ROS-JNK loop to induce cell death (Fan et al. 2011).

Although the above mentioned studies suggested a positive role of silibinin as anticancer mechanism, few study have also suggested a cytoprotective role of autophagy. Kauntz et al. showed that in human colon cancer cells, silibinin induced apoptosis *via* activation of both intrinsic and extrinsic pathways of apoptosis, but coincidentally it also induced autophagy in these cells. But when autophagic inhibitor was used, there is was a significant enhancement in cell death, suggesting a cytoprotective function of autophagy in these cells (Kauntz et al. 2011). Looking into the mechanism of silibinin mediated autophagy induction in cancer cells, most of the studies have attributed it to be orchestrated *via* ROS, and generation of ROS was further linked to major stress activated pathways in the cell including p53 induction, JNK activation and p38 signaling pathways.

EGCG or Green Tea Polyphenols

Green tea polyphenols (GTP) have been demonstrated to suppress tumorigenesis in several *in vitro* and *in vivo* models, and is one of the promising chemopreventive agents for human cancers (Yang et al. 2009). Green tea catechins (GTCs) including (-)-EGCG, (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC) were shown to suppress cell growth and induce apoptosis in various cell types in addition to their chemopreventive effect. Chung et al., demonstrated the role of GTCs in growth suppression, apoptosis induction, ROS formation and mitochondrial depolarization which was in order of ECG>EGCG>EGC>EC. Even though GTCs were able to induce apoptosis, there was no effect observed on members of Bcl-2 family as EGCG did not alter the expression of Bcl-2, Bcl-xL and BAD in prostate cancer cells (Chung et al. 2001). In a study done to evaluate the role of EGCG treatment on human monocytic leukemia U937 cells, it demonstrated an elevation of caspase 8 activity and its cleavage. The DNA ladder formation, a mark of apoptosis induction caused by the EGCG treatment was inhibited by the use of a caspase 8 inhibitor. These findings advocate the involvement of the Fas-mediated cascade in the EGCG-induced apoptosis in U937 cells (Hayakawa et al. 2001).

In hepatocellular carcinoma cells, EGCG inhibited the proliferation of cells by inducing apoptosis and blocking cell cycle progression in the G1 phase. Its effects

were partly brought about by significant increase in the expression of p53 and p21/WAF1 protein. An enhancement in Fas/APO-1 and its two ligand forms, membrane-bound Fas ligand (mFasL) and soluble Fas ligand (sFasL), as well as Bax protein was attributed for the apoptotic effect induced by EGCG (Kuo et al. 2003). EGCG-induced apoptosis in human prostate carcinoma LNCaP cells was mediated *via* modulation of p53 stability and p14 mediated down-regulation of murine double minute 2 (MDM2) proteins. EGCG was also shown to negatively regulate NF- κ B activity, thereby decreasing the expression of the pro-survival Bcl-2 proteins. Stabilization of p53 led to sustained transcriptional activation of its downstream targets Cip1/p21 and Bax. Thus, EGCG can regulate the expression of two major transcription factors in a manner that it shifts the balance towards triggering apoptosis rather than survival in these cells. The enhanced Bax/Bcl-2 ratio triggered the activation of initiator caspases 9 and 8 followed by activation of effector caspase 3 (Hastak et al. 2003). EGCG-induced apoptosis of pancreatic cancer cells was reported to be accompanied by growth arrest at an early phase of the cell cycle. In addition, EGCG upregulated Bax oligomerization and depolarization of mitochondrial membranes to facilitate cytochrome c release into cytosol. EGCG downregulated XIAP, an inhibitor of apoptosis protein which resulted in downstream caspase activation. Along with these effects, cells treated with EGCG elicited the production of intracellular reactive oxygen species (ROS), as well as the c-Jun N-terminal kinase (JNK) activation in pancreatic carcinoma cells (Qanungo et al. 2005). In head and neck carcinoma cells, EGCG increased Fas/CD95 expression along with a drastic decrease in the Tyr705 phosphorylation of STAT3. It is well known that STAT3 targets gene products such as Bcl-2, vascular endothelial growth factor (VEGF), Mcl-1, and cyclin D1 which are known to positively regulate cancer growth and progression. These molecules were also eventually down-regulated by EGCG treatment. The effect of EGCG was mediated *via* inhibition of STAT-3 activation was further validated by over expression of STAT3 in these cells, which led to resistance to EGCG treatment, suggesting that STAT3 could be a critical target of EGCG induced cell death in HNSCC cells (Lin et al. 2012a).

EGCG treatment is shown to cause the loss of mitochondrial membrane potential with the increase of ROS generation, p53 expression, Bax/Bcl-2 ratio, cytochrome c release, and cleavage of procaspase-3 and -9 and poly(ADP-ribose)-polymerase in cervical carcinoma cells (Singh et al. 2011). EGCG induced apoptosis of gastric carcinoma NUGC-3 cells has been found to be associated with lowered survivin and increased Bax and TRAIL expression and p73 activation. In this study, inhibition of p73 *via* siRNA diminished EGCG effects on survivin expression and cell viability. This study suggested that EGCG induces cell death in gastric cancer cells by apoptosis *via* inhibition of survivin which lies down-stream of p73 (Onoda et al. 2011). Analysis of the gene expression patterns of the androgen-independent prostate cancer cell line DU145, treated with EGCG revealed that EGCG modulated the expression levels by more than two-fold of 40 genes. These gene products were mainly involved in the functions of transcription, RNA processing, protein folding, phosphorylation, protein degradation, cell motility, and ion transport. Among them, inhibitor of DNA binding 2 (ID2), known as a dominant anti-retinoblastoma (Rb) helix-loop-helix protein, was found to be down-regulated 4-fold by EGCG treat-

ment. Overexpression of ID2 in DU145 cells reduced apoptosis and increased cell survival in the presence of EGCG, and its knock-down mimicked the apoptosis effect generated by EGCG treatment, although it was milder (Luo et al. 2010).

When NCI-H295 cells were treated with EGCG, the mitochondrial membrane potential decreased and intracellular free Ca^{2+} increased in a time-dependent manner. EGCG decreased the protein levels of Bcl-2, Bcl-xl, XIAP, cIAP, Hsp70 and Hsp90, but increased the protein expression of Bad, Bax, Fas/CD95, cytochrome c, Apaf-1, AIF, GADD153, GRP78, and cleaved caspase-3, -7, -8 and -9 (Wu et al. 2009). Overall, these studies suggest that EGCG has multiple targets for inducing cell death in various types of cancer cells.

Fisetin

Fisetin (3,7,3,4-tetrahydroxyflavone), a naturally occurring flavonoid commonly found in the smoke tree (*Cotinus coggygria*), is also found in fruits and vegetables such as strawberry, raspberries, apple, persimmon, grape, onion and cucumber. It exerts a wide variety of activities, including neurotrophic, anti-oxidant, anti-inflammatory and anti-angiogenic effects (Maher 2006). It has been reported to inhibit the proliferation of a wide variety of cancer cells, including prostate cancer (Haddad et al. 2010), liver cancer (Chen et al. 2002), colon cancer (Lu et al. 2005), and leukemia cells (Lee et al. 2002). In colon cancer cells HCT-116, fisetin induced apoptosis which involved enhanced ser15 phosphorylation of p53 and activation of caspase 3 and PARP. The study demonstrated the role of fisetin in modulating securin levels. Inhibition of securin resulted in potentiated response to fisetin induced apoptosis (Yu et al. 2011). In bladder cancer cells, fisetin induced apoptosis through mitochondrial pathway which was resultant of Bax up-regulation and cytochrome c release, especially at the higher (100 μM) dose (Li J et al. 2011).

TRAIL, which is an endogenous proapoptotic regulator of apoptosis, is mainly expressed by immune cells, and hence plays a very significant role in protecting the body against tumorigenesis. Studies done on evaluating the role of fisetin in cells which are resistant to TRAIL mediated apoptosis, showed that fisetin could sensitize these cells to TRAIL mediated apoptosis. Fisetin was also shown to increase the expression of TRAIL R1 receptor and down-regulate NF- κ B activity (Szliszka et al. 2011). When the role of Fisetin was explored in human breast cancer cells, it displayed differential effects showing better efficacy in MCF-7 cells than MDA-MB-231 cells without any cytotoxicity to MCF-10A cells, a non-tumorigenic breast epithelial cells. MCF-7 cells lack caspase 3, but fisetin could induce apoptosis in these cells, which was characterized by plasma membrane blebbing, mitochondrial depolarization and cleavage of caspase-7, -8, -9 and PARP. Interestingly, these cells did not show DNA fragmentation or phosphatidyl serine externalization which is one of the most common features of apoptosis (Yang et al. 2011).

In cervical carcinoma HeLa cells, fisetin triggered sustained activation of Erk1/2 together with cleavage of caspase 3 and PARP. Treatment with synthetic Erk1/2

inhibitor PD98059 also inhibited fisetin mediated activation of caspase-3 and -8 (Yang et al. 2012). Erk1/2 activation is typically associated with enhanced proliferation in cancer cells, but recent literature also show that sustained activation of Erk1/2 could lead to apoptotic cell death.

Fisetin is also shown to induce autophagic cell death in cancer cells. The autophagic effect of fisetin was shown to be exerted *via* inhibition of mTOR which is a component of the AKT signaling pathway and is known to play an important role in survival signaling. The study evaluated the effect of fisetin on human prostate carcinoma PC-3 cells which are null for PTEN and hence have an constitutively activated PI3K/AKT signaling. Fisetin down-regulated mTOR complexes and thereby induced autophagy in these cells. Many studies debate that induced autophagy could be a mechanism of cytoprotection, but this study showed that fisetin induced autophagy in PC-3 cells was not a protective mechanism (Suh et al. 2010).

Grape Seed Extract

Grape seed extract (GSE) is a complex mixture of polyphenols containing dimers, trimers, and other oligomers (procyanidins) of catechin and epicatechin and their gallate derivatives together known as the proanthocyanidins. GSE, a commonly used dietary supplement, is known to possess activities ranging from antiinflammatory, antioxidant, antibacterial and antiviral activities. Strong efficacies are recently reported against prostate, lung, breast, skin and other cancers. In prostate carcinoma LNCaP cells, as early as ~5 h treatment time with GSE, the cells get rounded in shape and got detached from culture dish suggesting an anoikis-like apoptotic cell death by reducing the cellular adhesion to extracellular matrix by reducing the level and activity of focal adhesion kinase (FAK). The study also showed that GSE could induce both anoikis and apoptosis involving decrease in FAK level and cleavage of caspases and PARP, and release of cytochrome c from the mitochondria to the cytosol, suggesting an activation of intrinsic pathway of apoptosis. However, the pan-caspase inhibitor z-VAD.fmk failed to completely attenuate GSE-induced apoptosis, implying that caspase-independent pathways were also involved in GSE-induced apoptosis (Kaur et al. 2006).

Of the non-cysteine proteases, AIF is thought to mediate apoptosis through caspase-independent pathway, where following a death stimulus, mitochondrial AIF is released into cytosol, which then translocates to nucleus and causes nuclear condensation followed by massive chromatin fragmentation and cell death. GSE phosphorylated ATM-Ser1981 in LNCaP cells, which might be responsible for p53 phosphorylation at Ser15. Following DNA damage, autophosphorylation of ATM at Ser1981 is required for the activation of ATM, which then phosphorylates downstream targets such as Chk2, p53 and H2A.X. Indeed, all these downstream targets of activated ATM were found to be phosphorylated following GSE treatment of LNCaP cells. GSE-induced apoptosis, cell growth inhibition, and cell death were found to be attenuated by the pretreatment with N-acetylcysteine, suggesting the in-

volvement of ROS in its apoptotic activity that might be a consequence of oxidative stress–caused DNA damage. The induction of ROS by GSE is also supported by another recent study showing that depending on its concentration, GSE itself could lead to the production of hydrogen peroxide (Kaur et al. 2006). In Caco-2 cells, GSE treatment inactivates the PI3K pathway leading to a concomitant decrease in Bad, cAMP response element-binding protein (CREB) and forkhead in rhabdomyosarcoma (FKHR) phosphorylation (Engelbrecht et al. 2007).

Another study done to assess whether the sources of GSE had any difference in effects, it revealed that irrespective of the sources, higher doses of GSE could induce apoptotic cell death in colon cancer cells. The apoptotic mechanism involved both intrinsic and extrinsic pathway, and release of AIF (Dinicola et al. 2010). This can be further validated by another study showing that GSE induced both intrinsic and extrinsic cell death in colorectal carcinoma cells involving caspase- dependent mechanism and release of AIF (Derry et al. 2012). One of the major reasons attributed to GSE-induced cell death was the generation of ROS which was independent of the p53 status of the colorectal carcinoma cells unlike what was observed in LNCaP cells (Derry et al. 2012).

The differential effect of GSE was also evident in head and neck carcinoma cells wherein it induced apoptosis in D-562 and FaDu cells but not in normal epidermal keratinocytes. The apoptotic response was characterized by activation of DNA damage checkpoint signaling ATM, ATR, Cdc-25C, which further led to the activation of caspase 8, 9 and 3 and inhibition of DNA repair BRCA1 and Rad 51, and enhanced ROS production (Shrotriya et al. 2012). In another study done with oral carcinoma cells with grape seed procyanidins (GSPs) showed that the effect on the cell was dependent on the p53 status of the cells. It was observed that in cells with wild type p53, GSP could induce mitochondrial dependent apoptosis whereas in cells which were mutant for p53, GSP induced cell cycle arrest but not apoptosis and no down-regulation of Bcl-2 protein was observed (Lin et al. 2012a). Overall, these studies suggest that GSE can selectively induce death of cancer cells involving ROS generation, extrinsic, intrinsic, caspase-dependent and –independent apoptotic pathways.

Other Natural Agents Inducing Cancer Cell Death

Other than these well studied natural plant molecules discussed above, many other compounds isolated from different plant sources have been shown to induce apoptosis in cancer cells (Table 1). Xanthohumol, a plant phytochemical was shown to act *via* extrinsic pathway in human colon carcinoma cells and activates FADD in the DISC that modulate release of caspase-8 and starts the induction of apoptosis (Pan et al. 2005). Zerumbone was also shown to enhance TRAIL-induced apoptosis through the up-regulation of DRs and the down-regulation of cFLIP (Yodkeeree et al. 2010). Zerumbone inhibited cell proliferation in liver cancer cells and induced apoptosis by decreasing the levels of anti-apoptotic protein, Bcl-2 and up-regulation

of pro-apoptotic Bax (Sakinah et al. 2007). Sanguinarine induced apoptosis in human leukemia cells by down-regulation of anti-apoptotic Bcl-2 and up-regulation of pro-apoptotic Bax expression (Han et al. 2008). Similarly, genistein decreases the ratio of Bcl-2/Bax and Bcl-xL/Bax in ovarian cancer cells which led these cells to programmed cell death and also enhances the phosphorylation and activation of p53 (Ouyang et al. 2009). Delphinidin significantly increased the levels of active caspases-3 and -9 in prostate cancer cells that leads to apoptosis, which was mediated *via* induction of Bax and inhibition of Bcl-2 protein (Hafeez et al. 2008). Garcinol changed the ratio of the anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins at the dose of 20 μM in colon cancer cells that resulted in cell apoptosis (Liao et al. 2005).

Resveratrol, a natural product from grapes and present in red wine, is well known to have anti-tumorigenic effect involving induction of apoptosis through activation of death receptor like Fas/CD95/APO-1 (Athar et al. 2006). Resveratrol induces alterations in mitochondria permeability pore transition, release of cytochrome c into the cytosol and conformational change in Apaf-1 which recruits the components of apoptosome complex and activate caspase cascade. Simultaneously, resveratrol promotes release of SMAC/DIABLO in cytosol which modulate IAPs activity and allow caspase-dependent apoptosis (Athar et al. 2010). Apigenin is known to induce ROS that promotes mitochondrial permeability and causes decrease in the mitochondrial Bcl-2 expression. Further, apigenin induced cytochrome c release and activated cleavage of caspase-3, 7, 8 and 9 that finally follow the apoptosis in prostate cancer cells; although in neuroblastoma, apigenin increased the intracellular free Ca^{+2} and allow these cells to start mitochondrial mediated apoptosis (Shukla and Gupta 2010). Human myeloid cells undergo apoptosis *via* activation of TNF up-regulation after the exposure of flavopiridol (Takada et al. 2008). Thus, many dietary and non-dietary phytochemicals have ability to induce death in cancer cells involving the known basic mechanisms of apoptosis induction and autophagy.

Summary and Conclusions

Induction of cell death remains one of the most desired effects of cancer therapy. Most of the therapeutically important agents work by induction of apoptotic, autophagic or necrotic death in cancer cells. These agents induce death *via* modulation of various signaling pathways activated in cancer cells. Enhancement of autophagic and apoptotic cell death induction by therapeutic agents especially selectively in cancer cells is important. One of the reasons for preferring natural agents with chemopreventive efficacy over the synthetic chemical agents, which induce cytotoxicity is that they are specifically more cytotoxic to cancer cells as compared to normal cells. One of the interesting phenomenon exhibited by these phytochemicals is that in normal cells most of these agents act as potent antioxidants which reduce generation of ROS, but majority of studies with these phytochemicals in cancer cells depict that these agents activate the mechanisms of cell death mainly by generation of ROS and hence creating a condition of oxidative stress. This duality in function which

leads to preferential cytotoxic effect towards cancer cells is the unique property of natural plant phytochemicals, and warrants further investigation at molecular levels.

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Dietary Phytochemicals Target Cancer Stem Cells for Cancer Chemoprevention

Dunne Fong and Marion M. Chan

Abstract Cancer is a multistep process involving genetic and epigenetic changes in the somatic genome. Genetic mutations as well as environmental factors lead to the initiation, promotion, and progression of cancer. Cancer progression ends in tumor metastasis to distant sites, and metastasis is the major reason for cancer patient deaths. Recent experimental evidence suggests the pivotal role of cancer stem cells. A tumor is heterogeneous and composed of different cell types. The cancer stem cells in the tumor have the capacity both to self-renew and differentiate to sustain the tumor. Features of cancer stem cells are described in this review, with an emphasis on the role that dietary phytochemicals may play in cancer chemoprevention. Ingredients in the diet can inhibit cancer cells and cancer stem cells. These compounds include curcumin from curry, epigallocatechin gallate from green tea, resveratrol from red wine, genistein from soy, sulforaphane from broccoli, and many others. Current research findings advocate the beneficial effects towards cancer chemoprevention via uptake of a combination of different dietary phytochemicals.

Keywords Phytochemicals • Cancer stem cells • Chemoprevention • Curcumin • Epigallocatechin gallate • Resveratrol • Genistein • Sulforaphane • Combination therapy

Cancer and Cancer Stem Cells

Biology of Cancer

A somatic cell undergoes changes in its genome and epigenome during its lifetime. If the accumulated changes involve regulation of cell growth and death, the

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resulting uncharted growth may alter the normal somatic cell to turn into a cancer cell. During the carcinogenesis process, there are malfunctions of genes, including gain of function of proto-oncogenes and loss of function of tumor suppressor genes.

Cancer cells exhibit hallmark alterations in their physiology, namely: (1) self-sufficiency of growth signals, (2) insensitivity to growth inhibitory signals, (3) evasion of programmed cell death, (4) limitless replicative potential, (5) reprogrammed cellular energetics, (6) induction of angiogenesis, (7) presence of tumor-promoting inflammation, (8) avoidance of immune destruction, (9) maintenance of genome instability and mutation, and (10) activation of tissue invasion and metastasis (Hanahan and Weinberg 2000, 2011).

Because cancer is a multistep process, it may take decades to develop. Carcinogenesis can be traced from its initiation, followed by promotion, progression, and metastasis. Accumulation of a handful of mutations (10–20) is adequate for cancer development. The cancer cell will then self-renew, migrate, and invade, as a clonal expansion in its complex tissue ecosystem (Greaves and Maley 2012). The niche or microenvironment surrounding the cancer cell can shape the path of carcinogenesis; as an example, the host immune system will attempt to destroy the developing cancer, although results may vary, as seen in the outcomes of cancer immunoediting (Schreiber et al. 2011). The tumor evolves from being benign to being malignant, and finally leads to the death of its host, the cancer patient. Investigators are interested in details of this process of carcinogenesis, and ways to halt its progression. Dietary phytochemicals have been shown to modulate the carcinogenetic procession.

Cancer Stem Cells

History and Definition

By histological examination, the tumor is heterogeneous, composed of tumor cells at various stages of cell differentiation, plus nontumor cells such as fibroblasts and immune cells. Thus, not all cells within a tumor are equal. Clonal evolution and competition within the tumor result in the dominance of the cell that is resistant to therapy and can both self-renew and differentiate its progeny cells. This observed tumor heterogeneity has been explained by the cancer stem cell (CSC) hypothesis. A workshop of the American Association for Cancer Research in 2006 settled with a consensus definition: CSC “is a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor” (Clarke et al. 2006). The putative CSC is also known as a tumor-initiating cell, a term preferred by some investigators, as seen in this example: “tumor-initiating cells (popularly known as cancer stem cells)” (Zhou et al. 2009). In this chapter, we use the popular term, CSC.

In a review published in 2001, the abstract starts with “stem cell biology has come of age” but ends with “cancer cells may include ‘cancer stem cells’—rare

cells with indefinite potential for self-renewal that drive tumorigenesis” (Reya et al. 2001). Research on various aspects of CSCs has been reported in the intervening years. In addition to research and review publications, CSCs are the topic of edited monographs. The first book dedicated to CSCs came out in 2009 (Bapat 2009); its editor was a scientist from India who first isolated human ovarian CSCs (Bapat et al. 2005). Other volumes subsequently appeared (Farrar 2010; Allan 2011; Scatena et al. 2012). One addressed CSC methods (Yu 2009). There are even Web-published volumes (Shostak 2011a, 2011b) and a series dedicated to cancer cells and CSCs (Hayat 2012). So, has CSC biology come of age?

In reality, the CSC hypothesis and the CSC concept have a long history. The subject has been billed as “old concepts, new insights” (Vermeulen et al. 2008) and “an evolving concept” (Nguyen et al. 2012). Linking stem cells and cancer, histological similarities were noted between embryonic and tumor tissues in the nineteenth century. This became the “embryonic rest” theory: cancers arise from cells with properties similar to those of early embryos. Later investigations concluded that cancer can be viewed as a “caricature” of normal development. The uncharted cell proliferation seen in cancer is the result of distortion of normal development, and a lack of coordination between growth and differentiation (Nguyen et al. 2012). In addition, knowledge on stem cell biology in normal tissues has been translated to the concept of CSCs in cancerous tissues.

Perhaps the best known normal stem cell is the hematopoietic stem cell (HSC). HSC has a hierarchy that permits an organized process of self-renewal and differentiation, giving rise to progeny of differentiated cell types including erythrocytes, macrophages, lymphocytes and many others. Immunologists have identified cluster of differentiation (CD) markers for the different cell types, and these cell surface antigens allow for the isolation of cells by methods such as fluorescence-activated cell sorting (FACS). The rare HSC exhibits the surface marker CD34, the member of a family of single-pass transmembrane sialomucin proteins. It should be noted that although CD34⁺ is seen in human HSC, the mouse equivalent is CD34⁻ or CD34^{low}. Thus, there are species differences in CD distribution.

In studying hematopoietic malignancies, in 1937, Jacob Furth and Morton Kahn provided the first quantitative assay for the assessment of the frequency of cancer cells maintaining the hematopoietic tumor. They showed that a single mouse leukemic cell was capable of transmitting the systemic disease when transplanted into a recipient mouse (as quoted by Clevers 2011). By definition, this has to be the CSC of murine leukemia.

Like its murine counterpart, the human CSC was first demonstrated in leukemia and then extended to solid tumors. Bonnet and Dick (1997) reported the presence of CD34⁺/CD38⁻ CSCs in human acute myeloid leukemia. Isolated patient CSCs will reconstitute the leukemia when transplanted in immunodeficient mice. Al-Hajj et al. (2003) reported the presence of CD44⁺/CD24^{-/low} CSCs in human breast cancer. They wrote: “As few as 100 cells with this phenotype were able to form tumors in mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors.” Singh et al. (2003) reported the presence of CD133⁺ CSCs in human brain cancer. They commented:

The increased self-renewal capacity of the brain tumor stem cell (BTSC) was highest from the most aggressive clinical samples of medulloblastoma compared with low-grade gliomas. The BTSC was exclusively isolated with the cell fraction expressing the neural stem cell surface marker CD133. These CD133⁺ cells could differentiate in culture into tumor cells that phenotypically resembled the tumor from the patient.

These initial studies on CSCs were expanded to many cancers in the following decade.

Biomarkers and Experimental Studies

Cellular antigens of CSCs can serve as biomarkers. The more common ones are CD24, CD34, CD44, and CD133 (Woodward and Sulman 2008; Keysar and Jimeno 2010). They are used in the isolation of CSCs from leukemia, breast cancer, and many other cancers. CD24 is a heat-stable antigen, a sialoprotein that acts as a ligand for P-selectin, thus enabling the cell to bind to platelets and protecting tumor cells in the blood stream. Although CD24 is negative in breast CSCs, it is present in ovarian CSCs (Gao et al. 2010). CD44 is a transmembrane glycoprotein which is a hyaluronic acid receptor. Besides breast cancer, CD44 is found in CSCs from pancreatic, gastric, head and neck, ovarian, and colon cancer. CD133, also named prominin-1, is a glycoprotein consisting of five transmembrane domains with a restricted expression within the plasma membrane protrusion sites. In addition to brain cancer, CD133 is found in CSCs from colon, liver, pancreatic, and prostate cancer (Alison et al. 2011; Zobalova et al. 2011; Hu and Fu 2012). It should be noted that these antigens are also present in the respective normal stem cells, although they have been used as biomarkers for their cancerous counterparts. Hence, the significance of CD24 and CD44 as CSC markers has been seen as “an enduring ambiguity” (Jaggupilli and Elkord 2012). Another important point is the lack of a single CD antigen common to all CSCs. However, a universal marker may be close at hand. In a recent drug screening study specifically targeting CSCs, dopamine receptors have been discovered as a biomarker for CSCs but not normal stem cells. Because only leukemic and breast CSCs have been tested so far, the novel biomarker requires confirmation and validation (Sachlos et al. 2012).

In addition to identification through biomarkers, CSCs can also be characterized by functional assays, namely, the detection of side population (SP) activity and the assessment of aldehyde dehydrogenase (ALDH) activity (Keysar and Jimeno 2010; Tirino et al. 2013). The SP assay measures the ability of cells to expel the fluorescent dye, Hoechst 33342, caused by the activity of ATP-dependent drug transporters on the plasma membrane, especially the ABCG2 transporter (Mo and Zhang 2012). As analyzed by flow cytometry, cells with fast drug exit form a side population, as opposed to the main population composed of the majority of cells. Initially identified in HSCs, SP has been found in many CSCs. Similarly, ALDH is a detoxifying enzyme and acts by the oxidation of aldehydes to carboxylic acids for further metabolism or liver exit. A fluorescent substrate assay using ALDEFLUOR, biodipy-aminoacetaldehyde (BAAA), allows for the isolation of ALDH⁺ cells. The ALDH

Table 1 Markers for Cancer Stem Cells. (Adapted from Woodward and Sulman 2008; Keysar and Jimeno 2010; Clevers 2011; Hu and Fu 2012; Sachlos et al. 2012 and other references cited in text)

Marker	Description	Examples of tumor types
CD24	Heat stable sialoglycoprotein	Ovarian cancer
CD34	Hematopoietic progenitor surface glycoprotein	AML (acute myeloid leukemia)
CD44	Hyaluronic acid receptor	Breast/colorectal/gastric/liver/head and neck/ovarian/pancreatic cancer
CD90	Thymocyte differentiation antigen-1 (Thy-1)	Glioma/liver cancer
CD133	Prominin-1	Brain/colorectal/lung/liver/ovarian/pancreatic/prostate cancer
CD326	Epithelial surface antigen (ESA) or epithelial cell adhesion molecule (EpCAM)	Colorectal/pancreatic cancer
ABCG2	ATP-binding cassette drug transporter	Brain/liver/lung/ovarian/pancreatic cancer
ABCG5	ATP-binding cassette drug transporter	Melanoma
ALDH	Aldehyde dehydrogenase	Breast/colorectal/lung/ovarian/pancreatic/prostate cancer
DR	Dopamine receptor	AML (acute myeloid leukemia)

assay was initially used to isolate HSCs and normal breast stem cells, and then extended to CSCs (Ginestier et al. 2007). Inasmuch as both SP and ALDH assays involve drug extrusion and metabolism, exhibiting these capacities indicates drug resistance of normal and CSCs. For example, SP and ALDH assays, in addition to specific biomarker (such as CD44 and CD133) screening, have been utilized for the identification of human ovarian CSCs (as summarized by Bapat 2010). However, it should be emphasized that these assays only enrich CSCs. The biomarker and functional assays may not characterize all the CSCs in the analyzed samples; therefore, additional CSCs not exhibiting the attribute being tested may still be present. For a list of biomarkers currently being used in the isolation of cancer stem cells, please see Table 1.

In addition to biomarkers and functional assays, another *in vitro* method to study CSCs is the use of serum-free spheres or spheroid cultures. Tissue culture cells are usually grown as a monolayer in a nutrient-rich medium containing fetal bovine serum as a source of the necessary growth factors and other components. However, in the absence of serum but in the presence of growth factors such as fibroblast growth factor (FGF) and epidermal growth factor (EGF), cells will grow as spheres in suspension in unattached/untreated tissue culture plastic dishes, flasks, or plates. Spheroid cultures mimic the three-dimensional nature of a tissue. Furthermore, it should be noted that oxygen will be less available to the cells located at the interior of a sphere. The hypoxic condition may modulate their differentiated state: towards stemness. Originally developed for neurobiological studies, the spheroid culture is used to “identify stem cells based on their reported capacity to evaluate self-renewal

and differentiation at the single-cell level in vitro” (Pastrana et al. 2011). Spheroid cultures have been developed for normal tissues and tumors (Chen et al. 2012a). When isolated, individual sphere-forming cells from a tumor will form secondary and tertiary spheres upon sequential subcultures; by operational definition these will be CSCs. However, there have been critiques on this spheroid assay. Spheres are prone to aggregate. Hence, cell density will influence clonality. The quiescent stem cell may be missed by this method (Pastrana et al. 2011). In fact, the quiescent nature of putative normal and CSCs has been utilized for their characterization. These cells are known as label-retaining cells, because they can retain labels such as the lipophilic dye PKH26, which is diluted in subsequent cell divisions but not so with slow-dividing cells (Pece et al. 2010; Xin et al. 2012).

From a tumor sample, putative CSCs may be characterized by the in vitro techniques discussed above. In addition to isolating CSCs from tumor samples, it is also possible to isolate them from established cancer cell lines (Drewa et al. 2011; Mather 2012). The advantage of the latter is the absence of nontumor cells as contaminants; the disadvantage is the additional accumulated changes during the long period of in vitro culture. As an example, our interest in CSCs and SP analysis led us to isolate SPs from the rat C6 glioma cell line, deriving putative CSCs from an established cell line (Fong et al. 2010). However, the C6 stemness state is dynamic: although SPs give rise to both SP and non-SP progenies, as expected, non-SPs can do the same (Fong et al. 2010; Fong and Chan 2012).

Complementary to in vitro studies of CSCs, in vivo models are available. The isolated cells are tested by their ability to initiate new tumor growth after xenotransplantation into immunocompromised mice (Cheng et al. 2010). Limiting dilution xenotransplantation yields an estimate of CSC abundance in the tumor sample; sequential transplantation yielding the original tumor will confirm the presence of CSCs. Different murine models with varying degrees of immunodeficiency have been applied to CSC studies. Examples include athymic nude mice, nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, and NOD/SCID interleukin-2 receptor gamma chain null (*Il2rg^{-/-}*) mice. For example, using the severely immunodeficient NOD/SCID *Il2rg^{-/-}* mice in single-cell transplants (in an appropriate extracellular matrix, Matrigel) from human melanoma samples, 27% of unselected melanoma cells develop tumors in mice (Quintana et al. 2008). Does this finding detect the abundance of human skin CSCs, whereas CSCs from other cancers are rare? There have been critiques on this xenotransplantation assay. Placing human cancer cells in immunodeficient mice is artificial because the human cancer cell will not encounter its normal host immune response. Therefore, results from this assay cannot reflect the physiological fate of the CSC in its native environment. Despite these objections, the limiting dilution murine in vivo xenotransplantation assay has been held as the “gold standard” for CSC identification (Ghiaur et al. 2012). However, other in vivo assays have been attempted for identifying CSCs. One example is the zebrafish model (Blackburn et al. 2011). Nonetheless, we found this quote: “despite being considered the gold standard assay for CSCs by many in the field, there is no reason to assume that growth in immunocompromised mice is in fact a relevant assay for CSC activity” (Ghiaur et al. 2012).

Controversy and Evidence

Even now, the CSC hypothesis is controversial. In 2009, one review asked: “Cancer stem cells: mirage or reality?” (Gupta et al. 2009a); another discussed: “Cancer stem cells and cancer nonstem cells: from adult stem cells or from reprogramming of differentiated somatic cells” (Trosko 2009); a third commented: “The investigation and study of cancer stem cells (CSCs) have received enormous attention over the past 5–10 years but remain topics of considerable controversy” (Rosen and Jordan 2009). Similar statements were made in 2013: “The investigation and study of CSCs have received enormous attention only over the past 5–10 years and remain topics of considerable controversy. Opinions about the validity of the CSC hypothesis, the biological properties of CSCs, and the relevance of CSCs to cancer therapy differ widely” (Tirino et al. 2013).

However, there is accumulating evidence favoring the CSC hypothesis. The CSCs may explain concepts of cancer and therefore are relevant to cancer therapy. The major supporting data are: (1) the presence of CSCs in minimal residual disease (MRD) and (2) the demonstration of CSCs in cell lineage tracing studies of murine tumors.

MRD is a term first used in leukemia, to denote the small numbers of leukemic cells that remain in the patient during or after treatment, when the patient is in remission (no symptoms/signs of disease). It is the major cause of relapse in cancer. MRD has been applied to solid tumors. If CSCs are resistant to therapy, they should be enriched after chemo- and radiotherapy and found in MRD. This has been shown to be the case. For example, resident breast CSC populations surviving conventional treatment (such as docetaxel) have been found to be enriched for CD44⁺/CD24^{-/low} breast CSCs that express epithelial-mesenchymal-transition (EMT)-associated genes (Creighton et al. 2009). Thus, the presence of CSCs after therapy predicts recurrence (Ghiaur et al. 2012).

Lineage tracing is a common technique for studying cell origins in developmental biology. Tracking cells with fluorescent proteins such as green fluorescent protein (GFP) in three different murine solid tumors, as reported in 2012, has been cheered as “resolving the stem-cell debate” (Gilbertson 2012; Graham 2012). In murine intestinal adenoma, 5–10% of the cells were CSCs, exhibiting the marker leucine-rich repeat-containing heterotrimeric guanine nucleotide-binding protein-coupled receptor 5 (Lgr5). The cell lineage tracing was made possible through the use of Cre-reporter at the *Rosa26-Confetti* locus, showing fluorescence (with four colors: green, red, yellow, blue) and even color conversion during tumor development. Lgr5 cells generated additional Lgr5 cells, as well as other adenoma cell types (Schepers et al. 2012). In murine papilloma, a benign skin tumor, 20% of cells were stem cells (tracked by yellow fluorescent protein) that divided twice a day whereas the others became terminally differentiated tumor cells (Driessens et al. 2012). In murine glioblastoma, a transgene was created to label both the quiescent adult neural stem cells and a subset of the endogenous glioma tumor cells (expressing GFP). The transgene also contained a viral thymidine kinase gene that could be targeted by the drug ganciclovir. Gliomas were treated with the drug temozolomide (TMZ);

but TMZ treatment alone led to the regrowth of a subpopulation of CSCs, that were then controlled by ganciclovir. TMZ-ganciclovir cotreatment impeded tumor development, by destroying both cancer cells and CSCs. Hence, this last study demonstrated the existence of murine glioma CSCs and their selective targeting (Chen et al. 2012b).

Resistance to Therapy and Stem Cell Pathways

With the assumption that findings in mice are extrapolatable to humans, the demonstration of CSCs in murine glioma and TMZ-ganciclovir cotreatment shows clinical relevance of CSCs. CSCs are resistant to therapy; they are or become chemo- and radio-resistant during or after therapeutic treatments (Donnenberg and Donnenberg 2005; Krause et al. 2011). These characteristics are due to the activity of drug transporters and metabolism enzymes, and a DNA repair system activated by genomic instability. CSCs may possess less reactive oxygen species (ROS), and thus are less susceptible to radiation therapy (Diehn et al. 2009). Depending on individual cases of cancer, CSCs may arise from either mutated normal stem cells, or dedifferentiated cancer cells exhibiting stem cell features. They display pathways of gene expression in common with those of normal stem cells. Therefore, thinking along therapeutic approaches, compounds targeting CSCs must be capable of differentiating them from the normal stem cells and sparing the latter, otherwise unforeseen problems with normal tissue homeostasis can occur.

Several signal transduction pathways are active in CSCs and may be amenable for intervention. The self-renewal pathways seen in CSCs relate to the expression of proteins involved in Hedgehog, Wnt, and Notch signaling. Additional pathways include PI3K and NF κ B pathways (Garvalov and Acker 2011; Alison et al. 2011, 2012; Hu and Fu 2012).

The Hedgehog (Hh) signaling pathway starts with a secreted morphogenetic factor. The term Hh comes from the fruit fly genetic mutation Hh that leads to spiny-looking larva; the gene is essential for arthropod segmentation and mammalian development. The mammalian Hh morphogen, as a ligand, binds to its receptor, Patched 1. This binding activates another plasma membrane protein, Smoothed, which eventually leads to activation of the transcription factor known as Gli (glioma).

The Wnt signaling pathway also starts with a secreted morphogenetic factor. The term Wnt comes from the fruit fly genetic mutation Wingless (Wg), which is important for arthropod polarity and segmentation, and the murine gene Integration 1 (Int1), a gene activated in breast cancer of mice infected with mouse mammary tumor virus. Wnt morphogen binds to its receptor, and after a series of intermediate steps, results in the mobilization of a cytoskeletal protein, *beta*-catenin, from the cytoplasm to the nucleus to activate its specific transcription factor known as lymphoid enhancer binding factor/T-cell factor (LEF/TCF).

Instead of secreted factors, the Notch signaling pathway starts with the membrane-associated Notch protein. The term Notch also comes from a fruit fly genetic

mutation, which results in a notch in the fly wing. The binding of Notch by its ligand, such as the membrane protein Delta from a neighboring cell, initiates the pathway. Notch protein undergoes limited proteolysis by the proteinase named *gamma*-secretase to yield the Notch intracellular domain (NICD), which mobilizes from the cytoplasm to the nucleus to activate its specific transcription factor named recombination signal binding protein for immunoglobulin kappa J (RBPJ), alias CSL (after the mammalian centromere promoter binding factor 1, CBF-1, the fruit fly suppressor of hairless mutation, and the nematode *lag-2* gene). Notch gene mutations/polymorphisms have been found in cancer patients, and may be involved in CSC chemoresistance (Crea et al. 2011).

The three signaling pathways initiated by Hedgehog, Wnt, and Notch are functional in embryonic stem cell development and may be dysregulated in CSCs. Activation of stem cell signaling pathways results in the expression of stemness genes (pluripotency) in CSCs. Examples are Oct4 (octamer-binding transcription factor 4, a homeodomain transcription factor), Nanog (a homeobox protein, another transcription factor), and Sox2 (sex determining region Y-box 2, a transcription factor with a high mobility group domain) commonly found in aggressive, poorly differentiated tumors (Ben-Porath et al. 2008).

Besides Hedgehog, Wnt, and Notch pathways, additional ones are PI3K and NF κ B (Alison et al. 2012). Phosphoinositide 3-kinase (PI3K) is linked to the mammalian target of rapamycin (mTOR) that relates to cellular energetics. The signaling pathway that leads to the activation of nuclear factor kappa B (NF κ B) is important for cytokine expression and the inflammatory response. Activation of these pathways results in the expression of stemness phenotype. The CSCs proliferate and differentiate, and may become resistant to treatments by chemicals and radiation. Molecules targeting these pathways, especially dietary phytochemicals, are discussed with respect to the CSCs.

Niche and Metastasis

Normal stem cells/CSCs reside in specific places. All cells are influenced by their surrounding environment, a local ecosystem. This concept is known as the stem cell microenvironment, or the stem cell niche. Thus, for “normal stem cells and cancer stem cells: the niche matters” (Li and Neaves 2006); and “location, location, location: the cancer stem cell niche” (Sneddon and Werb 2007).

Each resident cell is affected by its niche. The best characterized normal stem cell is the HSC. It behaves differently depending on the niche. In its bone niche, HSC is quiescent in the bone marrow. In its vascular niche, HSC undergoes proliferation and differentiation within the blood vessels. Similarly, the niche concept applies to the CSCs. In addition to the primary tumor site, there are secondary tumor sites after tumor metastasis. Each tumor site consists of a variety of cellular and extracellular components. In addition to cancer cells and CSCs, other cell types include blood and lymphatic endothelial cells, fibroblasts, adipocytes, and immune and inflammatory cells. These cells may secrete molecules that can either promote or inhibit the

tumor depending on changes in the microenvironment. The cytokines, chemotactic factors, and extracellular matrix produced by the various cells affect the behavior of cancer cells. Changes always occur within the tumor; the niche is dynamic. For example, there are cancer-associated fibroblasts and tumor-infiltrating macrophages. Separating the macrophage subsets as M1 and M2 with pro- and anti-inflammatory attributes, respectively, M1 protects against and M2 facilitates tumor development. Furthermore, the niche within the tumor is hypoxic. Hypoxia can be a determinant for CSCs, as CSCs may express hypoxia-inducible factors (Fábíán et al. 2013).

Biology of a tumor can only be understood by studying tumor cell heterogeneity and the niche that is constructed during the course of carcinogenesis. Hence, the situation becomes “the bed and the bugs: interactions between the tumor microenvironment and cancer stem cells” (Castaño et al. 2012). There is interdependence between the CSC and its niche. For example, paracrine signaling between carcinoma cells and mesenchymal stem cells (MSCs) has been reported (Li et al. 2012; Rasanen and Herlyn 2012). MSCs are recruited to the tumor niche. Carcinoma cells produce the cytokine interleukin-1, which induces the MSCs to produce the lipid molecule prostaglandin E₂ (PGE₂). PGE₂ induces changes in carcinoma cells by the activation of the *beta*-catenin signaling pathway and the formation of CSCs, via a process known as epithelial–mesenchymal transition (EMT). This example suggests that the niche allows CSC development.

Cells communicate within an epithelial cell layer. This comprises gap junctional intercellular communication (GJIC), an essential feature for cellular homeostasis (Trosko 2009). During EMT, epithelial cells undergo a transformation to become more mesenchymal in nature, from the tightly packed, highly differentiated, and immobile cells into the more loosely packed, less differentiated, and mobile cells. A special group of transcription factors is activated, including Snail and Slug (named after fruit fly zinc finger gene regulatory proteins). The adhesive protein E-cadherin (E for epithelial) is downregulated, leading to more motile cells. The absence of E-cadherin also allows for drug resistance, because its elevated expression has been associated with high drug sensitivity. Within the tumor, activation of the EMT program generates a reservoir of CSCs. EMT enhances cell survival by expressing genes that aid cells in avoiding apoptosis (programmed cell death), anoikis (cell death due to loss of cell adhesion), cellular senescence (the process of limited cell replication), and even host immune response (Tiwaria et al. 2012; Scheel and Weinberg 2012).

In addition to the contribution to the expression of stem cell phenotypes, EMT facilitates early stages of cancer cell invasion and metastasis. CSCs within the primary tumor leave this location to colonize distant sites; thus, metastasis is the major cause of human cancer deaths. The CSCs may be genetically unstable; multiple CSC clones may be present within the same tumor. The intrinsic genetic alterations keep the cells in the EMT and in the stemness state (Baccelli and Trumpp 2012). Once they have migrated to secondary sites, the cells may convert back to the epithelial state. Mesenchymal–epithelial transition (MET) can occur, but this reverse process is less well characterized. Nonetheless, there is phenotypic plasticity between the EMT–MET processes (Brabletz 2012).

Cancer, Cancer Stem Cells, and Mitochondria

Are there interactions among cancer, CSCs, and mitochondria? Almost all mammalian cells have mitochondria as their subcellular organelles, except mature erythrocytes. Mitochondria are essential for cell metabolism. In tumor cells, their mitochondria resist apoptosis-associated permeabilization. Tumor cell mitochondria also contribute to altered cell metabolism by stimulating cell growth and anabolic metabolism. Tumor cell metabolism can become a target for cancer treatment (Kroemer and Pouyssegur 2008).

An early discovery on tumor metabolism became known as the *Warburg effect*, after Otto Warburg, who reported the increased glycolysis in leukemic and solid tumor cells. The dominance of this work may have delayed investigations on cancer and mitochondria for some time. However, recent studies on mitochondrial enzymes and metabolism have shown that they play a significant pathogenic role in cancer (Scatena 2012).

When the cell experiences stress, its mitochondria respond. The rapid mitochondrial fission and fusion depend on GTPases, notably dynamin-related protein-1 (Drp-1) promoting fission and mitofusin-2 (Mfn-2) promoting fusion. The processes lead to elimination of damage (via fission) or compensation of damage (via fusion). Defective mitochondria are destroyed via autophagy (known as mitophagy). If all else fails, the stressed cell undergoes apoptosis (Youle and van der Bliek 2012).

That mitochondria fission is important for cancer has been demonstrated in lung cancer. Using human lung adenocarcinoma cell line A549 xenografts in nude mice, Mfn-2 gene therapy (with adenovirus vector) leads to smaller tumors as compared to control cancer cells. Similar tumor size reduction is also seen with the pharmacological approach using Mdiv-1, a selective inhibitor of mitochondrial division in yeasts and mammalian cells. Thus, impaired fusion and enhanced fission contribute to the proliferation/apoptosis imbalance in cancer (Rehman et al. 2012). Targeting cancer cell mitochondria has generated the development of a group of compounds known as *mitocans* (mitochondrially targeted anticancer drugs). One such example is *alpha*-tocopheryl succinate, an analogue of vitamin E (Neuzil et al. 2007).

Thus far, metabolic differences between normal and cancer cell mitochondria have been shown, and the latter may be a therapeutic target of cancer. Is there a relationship to CSCs? Breast CSCs, derived from the human breast cancer cell line MCF7, is susceptible to mitochondrially targeted vitamin E succinate (named MitVES) in vitro. Additionally, it has been proposed that the CSC marker CD133 may select the stem cells by both evading host immune response and by overcoming stress-induced apoptosis via a mitochondrial connection (Zobalova et al. 2011). In a study using the CD44⁺/CD24^{-/low} breast CSCs from MCF7 as xenografts to NOD/SCID mice, the mitochondrial targeting liposome carrying daunorubicin and quinacrine inhibits tumor development (Zhang et al. 2012a). In summary, CSCs can be targeted by mitochondrial drugs.

Chemoprevention and Phytochemicals

Occupational cancer was first identified by Percivall Pott in 1775: young chimney sweeps later got scrotal cancer because they worked naked to avoid dirtying their clothes. The remedy was to wear clothes during chimney work and to clean and bathe afterwards. Such measures were followed in Holland but not in England, resulting in individuals with less and more scrotal cancers, respectively. Prevention of cancer became a topic of interest; concepts of chemoprophylaxis and chemoprotection began being proposed. In 1976, Michael Sporn introduced the word “chemoprevention” while investigating vitamin A and its analogues, and this became the commonly accepted term (Sporn and Suh 2000; Lippman and Hawk 2009). Chemoprevention refers to the use of agents to inhibit, reverse, or delay tumorigenesis.

Fruit and vegetable intake has been part of the human diet. Being omnivorous, humans also consume various meats and other foods. Bioactive components from plants are nonnutrient dietary phytochemicals that may modulate gene expression and signal transduction pathways (Manson 2003; Surh 2003; Tan et al. 2011). Phytochemicals have been isolated and characterized from fruits such as grapes and apples, vegetables such as broccoli and onions, spices such as turmeric, and beverages such as green tea and red wine, as well as numerous other sources. Collectively, these compounds became known as chemopreventive agents. As chemopreventive agents, phytochemicals have been shown to interact with multiple cellular targets (Aggarwal and Shishodia 2006a; Lee et al. 2011) and even with the epigenome (Vanden Berghe 2012). With respect to chemoprevention, phytochemicals target inflammation (Murakami and Ohigashi 2007; Kim et al. 2009). This is important because inflammation may initiate cancer, which is the loss of cell cycle control (Meeran and Katiyar 2008). In addition, the chemopreventive phytochemicals are applicable to cancer therapy, because molecular mechanisms may be common to both chemoprevention and cancer therapy. Phytochemicals may modulate cancer development and even metastasis (Pan and Ho 2008; Gupta et al. 2010).

Among the diverse phytochemicals implicated in the inhibition of carcinogenesis, one major group comprises polyphenols. These compounds are virtually ubiquitous in plant materials and may occur at very high levels. Mostly synthesized from phenylalanine, plant phenolics serve as structural polymer components and are also responsible for ultraviolet light screening. They have antioxidant activity and act as signaling molecules. The most important role of plant phenolics may be in plant defense against pathogens and herbivore predators. With the discovery of health benefits of plant polyphenols, it has been proposed to optimize the phenolic content of the diet so as to obtain favorable consequences for the general health of the population (Parr and Bolwell 2000). Within the plant phenolics, one group of importance for chemoprevention is known as the flavonoids (Yao et al. 2011). Another group has been classified as phytoestrogens (Moutsatsou 2007). Phytochemical action on CSCs is discussed in the following sections. CSCs are known to be resistant to chemotherapy. Previously we reviewed “overcoming cancer drug resistance by phytochemicals” (Chan and Fong 2009). Relevant information on phytochemicals discussed earlier has been updated and incorporated in this chapter.

Dietary Phytochemicals Targeting Cancer Stem Cells

Dietary phytochemicals that target CSCs are timely research areas, as seen in the abundance of publications and reviews on the topic. Before covering the major phytochemicals involved, we discuss recent reviews on this subject.

Aptly the very first review we found has the title: “Targeting Cancer Stem Cells with Phytochemicals” (Kawasaki et al. 2008). Stem cell pathways are presented, as well as a list of clinical trials to evaluate various phytochemicals. A review on CSCs concluded that “dietary phytochemicals are natural products found in our diet and can be used to target cancer stem cells” (Subramaniam et al. 2010). Another review suggested the tissue-specific stem cells as a target for chemoprevention and concludes that “known chemoprevention agents including sulforaphane, vitamin D₃, curcumin, quercetin, genistein, vitamin E, and EGCG may attribute their success at least in part to regulating self-renewal and differentiation of tissue-specific stem cells” (Maund and Cramer 2011). In a chapter titled “Towards New Anticancer Strategies by Targeting Cancer Stem Cells with Phytochemical Compounds” (Tanveer et al. 2011), a suggestion was to target Oct4 function in CSCs by phytochemicals. In a review titled “Implications of Cancer Stem Cell Theory for Cancer Chemoprevention by Natural Dietary Compounds” (Li et al. 2011b), stem cell pathways and phytochemicals were discussed; the authors concluded that:

Naturally-occurring dietary compounds are advantageous in several aspects as chemoprevention agents: (1) they are present in commonly consumed food, which is readily available to most people in daily life; (2) they usually have very low or no toxicity, in contrast to most chemotherapy drugs; (3) many of these compounds have shown potential as an adjunct to chemotherapy drugs in some clinical trials. Although the reports were very limited for dietary compounds to inhibit CSCs, many of them have been shown to be involved in modulation of CSC self-renewal pathways.

The same conclusions were reiterated in their contributed book chapter (Li et al. 2011a).

In addition to dietary phytochemicals, CSCs can be targeted by phytochemical analogues (as reviewed by Dandawate et al. 2013), other natural products (Efferth 2012), and even Chinese herbs (Weber et al. 2012). To end this section, an appropriate quote is from a review titled “Cancer Stem Cells: Potential Target for Bioactive Food Components” (Kim et al. 2012): “Unquestionably, a diet-induced shift from deregulation to regulation in cancer stem cells could have profound influence on cancer relapses and therefore is of immense societal importance.”

Curcumin

Health Effects

Curcumin is a diferuloylmethane from the Indian spice turmeric. (See Fig. 1 for the chemical structure of curcumin.) It is responsible for the yellow color of curry

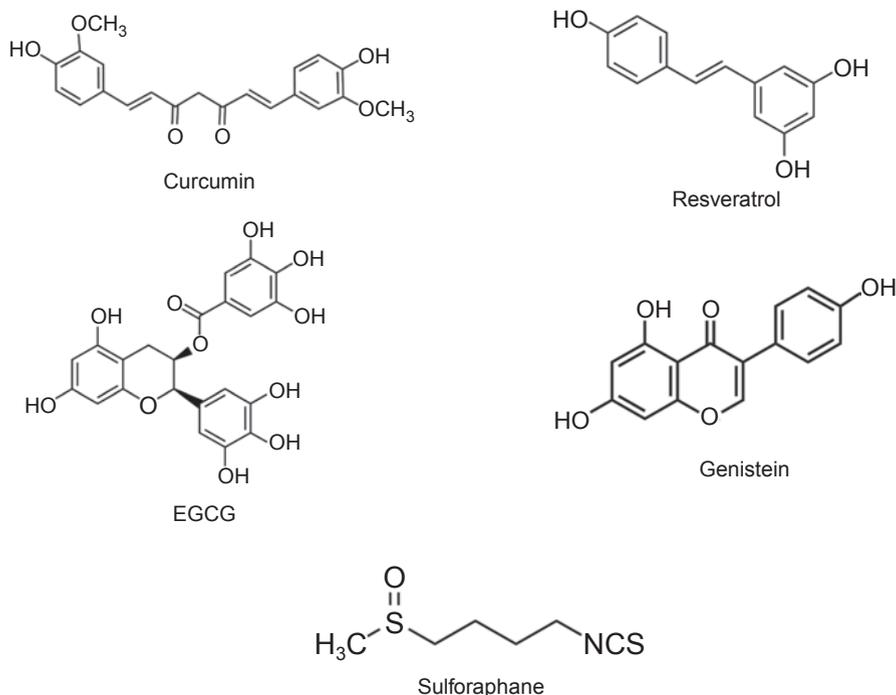


Fig. 1 Chemical structure of phytochemicals

powder. Turmeric is produced from the rhizome of the plant *Curcuma longa*. In addition to its use as a spice, turmeric has multiple applications in traditional Asian Indian medicine, ranging from treating insect bites to wound healing. Multiple health benefits have been associated with curcumin and turmeric, as summarized in a monograph (Aggarwal et al. 2007) and many reviews (Stimpakos and Sharma 2008; Goel et al. 2008). Multiple and diverse bioactivities have been attributed to curcumin, including cancer chemopreventive and anti-inflammatory activities (Bisht et al. 2010). Curcumin acts on many cellular targets (Ravindran et al. 2009). These include enzymes such as cyclooxygenase, lipoxygenase, and inducible nitric oxide synthase (iNOS); transcription factors such as NF κ B and activating protein-1 (AP1); cytokines such as tumor necrosis factor (TNF); and many gene products linked with cell survival, proliferation, invasion, and angiogenesis. For example, we found that curcumin inhibits NF κ B and TNF in a human monocytic cell line Mono Mac 6. With the endotoxin-induced murine sepsis model, curcumin ingestion by gavage induces bioactivity as shown by downregulating iNOS gene expression in the murine liver (Chan 1995; Chan et al. 1998). Synergistic effects between curcumin and chemotherapeutic drugs have been demonstrated: we have shown that curcumin enhances the cisplatin-mediated killing of the human ovarian cancer cell lines CAOV3 and SKOV3 in vitro (Chan et al. 2003; Chan and Fong 2007).

We end this section with a note on curcumin and patentability. In March 1995 the USPTO awarded the patent “Use of turmeric in wound healing” (US Patent 5,401,504) to Suman Das and Hari Har Cohly, with the University of Mississippi as assignee. The patent was withdrawn in August 1997, after protest from India’s Council of Scientific and Industrial Research (CSIR) and demonstration of the lack of novelty criterion required for patenting, inasmuch as turmeric has been used for centuries by the Indian population for wound healing (Jayaraman 1997). Whereas CSCs are drug- and radiation-resistant, curcumin is a chemosensitizer and radiosensitizer for tumors (Goel and Aggarwal 2010). However, we should also point out the “dark side of curcumin” (Burgos-Morón et al. 2010), as we note the potential of turmeric consumption in reducing the risk of cancer (Hutchins-Wolfbrandt and Mistry 2011).

Effects on Cancer Stem Cells

Effects of curcumin on CSCs have been shown. Using SP as selection for CSCs in the rat glioma cell line C6 *in vitro*, we found that curcumin (5 μM daily for 10 days via medium change) reduced SP, likely via the inhibition of drug transporter (Fong et al. 2010). Curcumin targets human glioblastoma CSCs *in vitro* (2 μM) and *in vivo*, the latter via intracranial implantation of human CSCs with intraperitoneal injections of curcumin (300 mg/kg every 3 days for 5 weeks); the investigators proposed the mechanism as induction of autophagy and promotion of differentiation (Zhuang et al. 2012). Curcumin targets CSCs of human esophageal squamous carcinoma cell lines *in vitro* (40–60 μM), showing a decrease in aldehyde dehydrogenase (ALDH) activity as well as CD44 and NF κ B expression (Almanaa et al. 2012).

Curcumin (30–60 μM) inhibits spheroid culture of human esophageal CSCs *in vitro* via the Notch signaling pathway (Subramaniam et al. 2012). Both human breast CSCs (from cell lines MCF7 and SUM159) and normal breast stem cells are targeted by curcumin (5–10 μM) together with another phytochemical piperine (from black pepper, 5–10 μM). There was a decrease in spheroid culture numbers as well as a decrease in ALDH activity (Kakarala et al. 2010). Both normal and CSCs were affected, therefore as a potential phytochemical for cancer therapy, its effect on normal stem cells must be clearly differentiated from CSCs.

Curcumin can complement other drugs. *In vitro*, cotreatment of curcumin (20 μM) and FOLFOX/FUOX (50 μM fluorouracil and 1.12 μM oxaliplatin) for 5 days inhibited the spheroid cultures of human colon CSCs from the cell lines HCT116 and HT29 (Yu et al. 2009). Induction of apoptosis in human brain tumor stem cells (commercially available from Celprogen) *in vitro* has been shown with cotreatment of curcumin (20 μM) and paclitaxel (10 nM) (Hossain et al. 2012). These results show the feasibility of combining conventional drugs with phytochemicals to target CSCs in clinical cancer.

However, the solubility of curcumin is a concern. To overcome this hurdle, various carriers have been developed. A polymeric nanoparticle formulation of curcumin, nanocurcumin trademark as NanoCurc (5–20 μM), induced apoptosis and reduced the CD133⁺ stem cells in malignant brain tumors *in vitro*

(Lim et al. 2011). A hyaluronic acid (HA)-based nanogel-curcumin conjugate, using cholesteryl-HA, had 8 times higher cytotoxicity than curcumin alone towards human pancreatic CSCs expressing CD44 (Wei et al. 2013). The authors also reported similar effects with nanogel-drug conjugates of two other compounds targeting CSCs, namely etoposide and salinomycin. A novel micelle formulation of curcumin, composed of stearic acid-g-chitosan oligosaccharide (CSO-SA) as a polymeric, biocompatible, and biodegradable drug carrier, inhibited CD44⁺/CD24⁺ colorectal CSCs in vitro and in vivo (nude mice xenografts), the latter at a dose of 2 mg/kg daily for 14 days in a 15-day experiment (Wang et al. 2012a). Another approach for curcumin solubility is to synthesize analogues. Difluorinated-curcumin (CDF) inhibited spheroid cultures of human colon CSCs from the cell line HCT116 (at 2–8 μM) in vitro (Kanwar et al. 2011). CDF (2 μM) plus FOLFOX/FUOX (100 μM fluorouracil and 2.5 μM oxaliplatin) inhibited the growth of the HT-29 colorectal cancer cell line CSCs in vitro via downregulation of microRNA-21 (miR-21) and promotion of differentiation (Yu et al. 2013). GO-Y030, a monoketone analogue of curcumin (Sato et al. 2011), inhibited human colorectal CSCs from the cell lines HCT116 and SW480 in vitro (induced apoptosis at 0.5–2.5 μM) and in vivo (NOD/SCID mice xenografts), the latter at a dose of 50 mg/kg daily for 14 days in a 29-day experiment (Lin et al. 2011). These results show that human CSCs can be targeted by curcumin and analogues in vivo using mouse models.

Resveratrol

Health Effects

Resveratrol is a stilbene (3,4',5-tri-hydroxy-trans-stilbene to be exact) from red wine, grapes, peanuts, and pine nuts. (See Fig. 1 for the chemical structure of resveratrol.) Red wine is made from whole grapes (including grape skin) from the fruits of the grape vine plant *Vitis venifera* and related species. Resveratrol is present in red wine because the molecule is associated with the skin portions of the fruit to fight against fungal infections of the plant. It is an example of the phytoalexins, compounds produced by plants against pathogens. Similar to plant fungal pathogens, we demonstrated it is active against human dermal fungal pathogens: resveratrol may be useful for treating athlete's foot (Chan 2002). Health benefits associated with resveratrol are summarized in a monograph (Aggarwal and Shishodia 2006b) and many reviews (Athar et al. 2007; Gescher 2008; Harikumar and Aggarwal 2008; Kundu and Surh 2008; Saiko et al. 2008). It is "one molecule, many targets" (Pirola and Fröjdo 2008). Resveratrol's recent claim to fame involved its action as an activator of sirtuin deacetylases, leading to lifespan extension from organisms as diverse as yeasts, nematodes, fruit flies, and fish (Baur and Sinclair 2006). Resveratrol's prior claim to fame involved a phenomenon known as the "French Paradox" (Opie and Lecour 2007), referring to the observation that the French benefit from a relatively low risk of developing cardiovascular disease despite a diet that is high in saturated fat.

Resveratrol improves general health in mice and seems to delay their ageing parameters (Barger et al. 2008; Pearson et al. 2008). In this sense, it mimics a phenomenon known as caloric restriction, a method that retards aspects of the ageing process in mammals (Maxmen 2012). Indeed, it has anti-inflammatory activity (Bisht et al. 2010) and shows health benefits, especially cardioprotection and cancer chemoprevention. With respect to the French Paradox, it is believed resveratrol and alcohol in red wine may be part of the reason. We have shown a synergistic effect between resveratrol and alcohol in the inhibition of iNOS (Chan et al. 2000). Moderate consumption of red wine may be beneficial to human health.

We end this section with a commercial note. In April 2008, the FDA granted Orphan Drug Status to resveratrol, for the treatment of MELAS syndrome: mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes, caused by mitochondrial DNA mutations. The sponsor was Sirtris Pharmaceuticals, a company in Massachusetts with expertise in resveratrol and its analogues (SRT501 was their resveratrol formulation). Orphan drug designation provides a company with seven years of marketing exclusivity. In late April 2008, Sirtris was purchased by GlaxoSmithKline for \$ 720 million. In December 2010, Sirtris announced that the clinical trial of SRT501 was halted but studies with other synthetic resveratrol-mimicking drugs would continue. Currently, the role of resveratrol as an antiageing molecule seems to be in doubt (Wade 2011). However, we must emphasize that resveratrol possesses cancer chemopreventive properties. The difficulty seems to be deciding on dietary-relevant doses (Scott et al. 2012).

Effects on Cancer Stem Cells

Effects of resveratrol on CSCs have been shown. These include CSCs from acute myeloid leukemia (AML), brain, breast, head and neck, and pancreatic cancer. Inasmuch as resveratrol has multiple cellular targets, several of the cellular targets in CSCs have been revealed. In AML patients there is an increase in the cytokine interleukin 6 (IL-6) in their plasma. IL-6 induced activation of the Sonic Hedgehog signaling pathway has been shown in the human HL-60 cell line, and resveratrol (25 μM) inhibits this activation and decreases cell viability (Su et al. 2013). In malignant brain tumor medulloblastoma CSCs that were grown in spheroid cultures, resveratrol (100 μM) inhibited proliferation in vitro and enhanced radiosensitivity (Lu et al. 2009). In primary central nervous system atypical teratoid/rhabdoid tumors, resveratrol (150–200 μM) induced apoptosis of the isolated CD133⁺CSCs in vitro and also enhanced radiosensitivity. The in vitro resveratrol-treated and irradiated CD133⁺CSCs resulted in better survival of SCID mice in a 14-week xenograft experiment (Kao et al. 2009). In glioblastoma multiforma, comparison of CD133⁺ and CD133⁻ cells has shown the involvement of signal transducer and activator of transcription 3 (STAT3). Resveratrol acts via inhibiting the STAT3 cytokine signaling pathway. CSCs treated in vitro with resveratrol (200 μM) and irradiation resulted in better survival of SCID mice; this effect of resveratrol can be mimicked with small hairpin (sh) RNA of STAT3 (Yang et al. 2012a).

Moving from the central nervous system to other organs, breast CSCs have been targeted by resveratrol. Using CD44⁺/CD24⁻ CSCs isolated from cell lines MCF7, MDA-MB231, and 231LM, resveratrol (50–100 μ M) inhibited spheroid cultures by inducing apoptosis. The mechanism involved the action of resveratrol on lipid synthesis, by its downregulation of fatty acid synthase (FAS). Luciferase-expressing CSCs from MDA-MB231 showed smaller-sized tumors in nude mice xenografts with resveratrol administration (22.4 mg/kg via oral gavage or intraperitoneal injection) every 2 days in a 28-day experiment (Pandey et al. 2011). Similar results have been reported by the same investigators using CSCs isolated from another human breast cancer cell line, MCF10DCIS.com (Pandey et al. 2012). In human head and neck cancer, resveratrol (25–100 μ M) eliminates aldehyde dehydrogenase (ALDH) activity, spheroid formation, and epithelial mesenchymal transition (EMT) in vitro, as well as reduces tumor xenograft volume and weight in vivo in immunodeficient mice (40 mg/kg daily for 20 days) (Hu et al. 2012). Human pancreatic CD133⁺/CD44⁺/CD24⁺ CSCs formed spheroid cultures and expressed stemness genes such as Oct4, Nanog, and Sox2. These activities were inhibited by resveratrol (10–30 μ M) in vitro. In a special transgenic mouse model, KRAS^{G12D}, the mice were designed to develop pancreatic ductal adenocarcinoma. The increase in size of the pancreas was inhibited by resveratrol (40 mg/kg) administered 5 days a week for 10 months. In addition to inhibiting the stemness genes, alias pluripotency maintaining factors, resveratrol inhibited EMT (Shankar et al. 2011). In summary, resveratrol aims at different cellular targets of CSCs, depending on the cancer type.

Epigallocatechin Gallate

Health Effects

EGCG is a polyphenol from green tea. (See Fig. 1 for the chemical structure of EGCG.) Tea is made from leaves of the plant *Camellia sinensis*, originally from China. EGCG is the major green tea polyphenol, accounting for 30–40% dry weight of the water-extractable material of green tea. It is converted to dimers and polymers during black tea production. Tea is one of the most popular beverages consumed in the world. Multiple health benefits have been associated with EGCG and tea consumption (Zaveri 2006; Yang et al. 2007; Khan and Mukhtar 2008). These include cancer chemoprevention, as well as the reduction of atherosclerosis, hypercholesterolemia, Alzheimer's and other ageing-related diseases. EGCG targets multiple signaling pathways. For example, we reported EGCG inhibition of iNOS both at the gene expression level and at the enzyme activity level (Chan et al. 1997). EGCG acts synergistically with cisplatin to kill ovarian cancer cells. Even in the cisplatin-resistant cell line C200, enhanced cell death by cisplatin was seen in the presence of EGCG in cell culture (Chan et al. 2006).

We end this section with a commercial note. In October 2006, the FDA approved an extract of green tea as a prescription drug for topical treatment of genital warts (Gross et al. 2008). The extract is Polyphenon E (Veregen) ointment (from a German biotech company MediGene and its marketing partner Bradley Pharmaceuticals,

Inc.). This was the first prescription botanical (herbal) drug approved by the FDA both under its current regulatory policy for botanical products as well as the FDA's new Drug Application Process (Blumenthal 2007).

Effects on Cancer Stem Cells

Effects of EGCG on CSCs have been shown. Actually, early reports used normal stem cells. Spheroid cultures for CSCs followed the initial work with normal neurospheres. In rat neurospheres, EGCG (10–50 $\mu\text{g/ml}$) and related compounds inhibited neurosphere adhesion and migration (Chen et al. 2003). Extending to CSC spheroid cultures, epigallocatechin (EGCG without the gallate moiety) and polyphenon 60 (characterized green tea extract, 10 μM) prevented cell shedding from mouse mammary cancer mammospheres in vitro. The mechanisms proposed are decreased ROS production and downregulation of matrix metalloproteinase-9 (Günther et al. 2007). EGCG (50 μM) inhibited spheroid cultures of human neuroblastoma BE(2)-C cells in vitro. These CSCs exhibit ALDH activity as well as the expression of stemness genes such as Oct4 and Nanog (Nishimura et al. 2012).

Both EGCG and its analogues act on CSCs. Several chemically synthesized analogues (5–40 μM) target human breast CSCs cultured as mammospheres from the cell line MDA-MB231. The mechanism has been attributed to activation of the AMP-activated protein kinase (AMPK) pathway, because a known activator of this pathway, metformin (5–10 mM), behaves in the same way (Chen et al. 2012c).

Synergistic effects of EGCG and another phytochemical, quercetin, on CSCs have been investigated. In human pancreatic CSCs, EGCG (20–60 μM) inhibited spheroid cultures, EMT, and the Hedgehog pathway in vitro. This activity was synergistic with quercetin (20 μM), as shown by increased apoptosis (Tang et al. 2012). In another publication, the same investigators also reported similar effects on human prostate CSCs (Tang et al. 2010). In summary, EGCG can act on CSCs either alone or in concert with another phytochemical.

Genistein

Health Effects

Genistein is an isoflavone (4,5,7-trihydroxyisoflavone to be exact) from soybeans. (See Fig. 1 for the chemical structure of genistein.) Soybeans are seeds from the legume plant *Glycine max*, originally from China, although China currently imports soybeans from the United States. It is believed that the isoflavone in soy-rich foods contributes to lower rates of prostate and breast cancers observed in China and Japan as compared to Western countries. Genistein is a molecule with many cellular targets, including inhibition of the NF κ B pathway, modulation of the cell cycle, and induction of apoptosis (Banerjee et al. 2008). In addition, genistein has been classified as a phytoestrogen. However, it is lipid soluble and exhibits low oral

bioavailability in pharmacokinetics studies (Yang et al. 2012b). Furthermore, there is a “dark side” to “the whole soy story” (Daniel 2005). Soybeans contain proteinase inhibitors that can be “toxic” to digestion. Also, people may develop allergic reactions to soy.

We end this section with an interesting demonstration of the transgenerational effect of genistein. The agouti mouse model, as noted by color change in the fur, is useful as an epigenetic biosensor for nutritional and environmental alterations on the fetal genome (Dolinoy 2008). Maternal dietary supplementation with genistein (250 mg/kg in the diet) led to DNA hypermethylation in the embryo and the methylation state was maintained until adulthood. Thus, phytochemicals such as genistein modify the epigenome, and the effect starts early in embryogenesis (Dolinoy et al. 2006).

Effects on Cancer Stem Cells

Effects of genistein on CSCs have been shown. Genistein was reported as a protein tyrosine kinase inhibitor. In an in vitro study on chronic myelogenous leukemia in 1996, the stem and progenitor cells were more sensitive to genistein (200 μM) than the normal progenitor cells (Carlo-Stella et al. 1966a, 1996b). In human prostate cancer, the CSCs from the cell line PC3 had been targeted by genistein (15 μM) in vitro, which aided in the death of these CSCs by docetaxel. In this work, the CSCs have been named “the Achilles’ heel of cancer” (McCubrey et al. 2011). Another study on prostate cancer extended its focus to in vivo xenografts, and discovered the cellular target as inhibiting the Hedgehog pathway (Zhang et al. 2012b). Genistein (30 μM) inhibited spheroid cultures of prostate CSCs from the cell lines 22RV1 and DU145, and inhibited tumor development in vivo in nude mice (twice weekly of genistein and/or docetaxel at 10 mg/kg) in a 28-day experiment. Although the CSCs were resistant to docetaxel, genistein plus docetaxel decreased tumor volume more than genistein alone. Another interesting report involved the feeding of genistein to mice, “at concentrations present in soy protein isolate (250 mg/kg food;” Montales et al. 2012). Sera from the genistein-fed animals were used to treat human breast CSCs from the cell lines MCF7 and MDA-MB231. When 1–5% of these sera was added to the medium, spheroid formation was inhibited, suggesting “factors found in sera of mice consuming” genistein were active against CSCs. Molecular details of the factors were not characterized. However, this last investigation is interesting with respect to chemoprevention: genistein in the diet leads to the animals having factors in the circulation that inhibit CSCs.

Sulforaphane

Health Effects

Sulforaphane, alias 1-isothiocyanato-4-methylsulfinylbutane, is an isothiocyanate found in brassicaceous vegetables. (See Fig. 1 for the chemical structure of

sulforaphane.) Broccoli, cabbage, cauliflower, Brussel sprouts, and kale are cultivars of the plant known as *Brassica oleracea*. These common vegetables belong to the plant order Brassicales. An older name is Cruciferae; they are also named crucifers or the cabbage family. The wild cabbage originally came from Europe. Broccoli and related vegetables contain many phytochemicals (Latte et al. 2011). In addition to sulforaphane, there are other compounds, such as indole-3-carbinol (I3C) and quercetin. Sulforaphane is released from the plant cell wall by activity of the enzyme myrosinase via cooking or chewing. The compound has multiple cellular targets: as an antioxidant, an activator of phase II detoxification enzymes, an anti-inflammatory agent, an inducer of apoptosis, and other roles (James et al. 2012). Broccoli has been used, as an example, for a “nutritional genomics” approach “in conveying to the consumer” that “the dietary regime being marketed will positively affect their health” (Ferguson and Schlothauer 2012).

We end this section with a quote on broccoli from a former US president (Dowd 1990). In March 1990, President George H. W. Bush, in response to queries about a broccoli ban he had imposed upon Air Force One, said: “I do not like broccoli. And I haven’t liked it since I was a little kid and my mother made me eat it. And I’m President of the United States, and I’m not going to eat any more broccoli!” In fact, the president might be a “supertaster.” People who find Brussel sprouts unbearably bitter carry a variant of the bitter taste receptor (T2R38) which results in their enhanced innate immunity (Lee et al. 2012a).

Effects on Cancer Stem Cells

Effects of sulforaphane on CSCs have been shown. Sulforaphane has been shown to inhibit human breast CSCs from the cell lines MCF7 and SUM159 in vitro and in vivo (Li et al. 2010). Spheroid cultures were inhibited (10–20 μM), as well as ALDH expression. In NOD/SCID mouse xenografts, two successive generations elegantly showed a decrease in tumor volume and an increase in survival (50 mg/kg daily for 14 days) in a 35-day experiment. Sulforaphane inhibited spheroid cultures in human pancreatic CSCs from the cell line MIA-PaCa2 in vitro (20 μM) and in vivo (Rausch et al. 2010). Sulforaphane inhibits renewal of pancreatic cancer stem cells in vitro (20 μM) by inhibiting the Sonic Hedgehog signaling pathway (Rodova et al. 2012). In nude mice xenograft experiments, sulforaphane (60 mg/kg) or co-treatment with the multikinase inhibitor sorafenib (3 mg/kg), daily for 3 days in a 5-day experiment, reduced tumor size via inhibition of the NF κ B pathway (Rausch et al. 2010). The same research group also extended their report on pancreatic CSCs to include results on prostate CSCs derived from the cell line DU145 (Kallifatidis et al. 2011). Sulforaphane was added in vitro to taxol, cisplatin, gemcitabine, fluro-uracil, or doxorubicin. Targeting was specific for CSCs, because normal cells (primary human fibroblasts and umbilical vein endothelial cells) were not affected. In vivo nude mice xenografts for pancreatic CSCs used sulforaphane in combination with gemcitabine (3 mg/kg consequently for 3 days; treatment started after tumor development was observed, in a 40-day experiment). In a different experiment, the

pancreatic CSCs had been pretreated with the sulforaphane–gemcitabine combination before xenograft. Both protocols resulted in decreased tumor volume (Kalifatidis et al. 2011). In fact, sulforaphane alone could reduce human pancreatic CSC growth in immunodeficient mice, through inhibition of the Sonic Hedgehog signaling pathway (20 mg/kg once a day for 5 days in a 6-week experiment; Li et al. 2013). In addition to solid tumors, sulforaphane enhancement of drug efficacy has been reported for leukemia. The CD34⁺ chronic myeloid leukemia CSCs previously resistant to imatinib were killed by a combination of imatinib (0.1–1 μ M) and sulforaphane (20–30 μ M) in vitro, via activation of the Wnt pathway and ROS induction (Lin et al. 2012).

Other Dietary Phytochemicals

Many phytochemicals may serve as chemopreventive agents. In some cases, their activities on CSCs have been investigated. In addition to sulforaphane, another important phytochemical found in the brassicaceous vegetables is indole-3-carbinol (I3C). I3C is a derivative of glucobrassicin, a secondary plant metabolite. I3C can be further metabolized into a dimer (3,3''-diindolmethane, DIM). I3C, DIM, and related synthetic analogues are potential agents of chemoprevention and cancer therapy (Weng et al. 2008; Safe et al. 2008). DIM inhibited spheroid culture formation, for CSCs from human and mouse cancer cell lines (the human non-small-cell lung carcinoma H460 and the murine melanoma B16/F10) in vitro. DIM-treated melanoma CSCs showed smaller tumor volume in vivo (Semov et al. 2012).

Quercetin is a flavonol found in common fruits and vegetables such as apples, broccoli, and onions. It has antioxidant activity and has been reviewed as safe in vivo, although mutagenic in vitro (Harwood et al. 2007; Boots et al. 2008; Gibellini et al. 2011). We reported that quercetin sensitized the killing by cisplatin of the human ovarian cancer cell line SKOV3 (Chan et al. 2003). Quercetin–EGCG's synergistic activity on human pancreatic CSCs has been discussed (Tang et al. 2011). In fact, quercetin by itself inhibited spheroid cultures of pancreatic CSCs, and it exhibited a synergistic effect with sulforaphane. In nude mice xenografts, quercetin and sulforaphane decreased tumor size (Zhou et al. 2010). Quercetin also targets the heat shock protein Hsp27. In human breast CSCs, inhibition of spheroid cultures by the Hsp90 inhibitor geldanamycin was enhanced by quercetin (Lee et al. 2012b). In human lung CSCs from the cell line A549 as xenografts to NOD/SCID mice, tumor volume was decreased by treatment with quercetin, cisplatin, and gemcitabine (Hsu et al. 2011a).

Cucurbitacin I, a cell-permeable triterpenoid compound, is found in members of the Cucurbitaceae family (cucumber, squash, melons, pumpkins) as well as brassicaceous vegetables. Investigators from Taiwan showed its effect on CD44⁺/ALDH⁺ CSCs from head and neck squamous cell carcinoma (Chen et al. 2010), as well as CD133⁺ CSCs from non-small-cell lung cancer (Hsu et al. 2011b), thyroid cancer (Tseng et al. 2012), and pediatric brain cancer medulloblastoma (Chang et al. 2012). Its cellular target is the STAT3 cytokine pathway.

Lupeol is another triterpene found in fruits and vegetables. A research group in Hong Kong demonstrated its effect on CSCs from liver cancer (Lee et al. 2011). Its

cellular target is the phosphatase and tensin homologue (PTEN)-protein kinase B (Akt)-ABCG2 pathway.

Extending from purified phytochemicals to plant extracts, a blueberry preparation fed to mice has been shown to be effective towards CSCs. Sera from blueberry-fed animals inhibited spheroid cultures of human breast CSCs (Montales et al. 2012). A pomegranate extract also inhibited mouse mammary CSCs in vitro (Dai et al. 2010)

In summary, individual phytochemicals and plant extracts from fruits, vegetables, spices, and beverages may be harnessed to inhibit CSCs, but their cellular targets may vary depending on the cancer type. A list of probable molecular targets for phytochemicals in CSCs can be found in Table. 2.

Other Molecules Targeting Cancer Stem Cells

In addition to dietary phytochemicals, many other molecules target CSCs. We start with compounds isolated from plant species. Parthenolide (PTL), from the feverfew plant (*Tanacetum parthenium*), is commonly used in herbal medicine. PTL targets human acute myelogenous leukemia stem cells (Guzman et al. 2005). As an inhibitor of NFκB, it inhibited human breast CSCs from the cell line MCF7 (Zhou et al. 2008). Screening in silico for compounds with similar effects on gene expression as PTL has revealed others, including celastrol, found in thunder god vine (*Tripterygium wilfordii*) and used in Chinese herbal medicine (Hassane et al. 2008). Another compound, berberine, from plants such as goldenseal (*Hydrastis canadensis*) and the Chinese goldthread (*Coptis chinensis*), also used in herbal medicine, targets MCF7 SP cells (Kim et al. 2008). MCF7 SP cells were also inhibited by the alkaloid oxymatrine, from the Chinese herbal medical plant *Sophora flavescens* (Zhang et al. 2011). Eriocalyxin, an analogue of the natural diterpene from the Chinese herbal medical plant *Isodon eriocalyx*, inhibited human ovarian CSCs via NFκB inhibition (Leizer et al. 2011). Cyclopamine from corn lily (*Veratrum californicum*) targets glioblastoma CSCs by inhibiting the Hedgehog pathway (Bar et al. 2007). However, it should be noted that cyclopamine is a teratogen that causes holoprosencephaly (cyclopia). Silybin, alias silibinin, is a flavonoid compound from the herbal medical plant milk thistle (*Silybum marianum*). The phytochemical targets colorectal CSCs (Wang et al. 2012b). Butein is a chalconoid compound from the Chinese lacquer tree (*Toxicodendron vernicifluum*) with aromatase-inhibiting effects. It targets human breast CSCs (Ciocce et al. 2010). Gossypol is a polyphenol from the cotton plant (genus *Gossypium*) and at one time had been tested as a male contraceptive. It targets prostate CSCs via activation of the p53 gene regulatory protein (Volate et al. 2010). From the above studies, it is obvious that many phytochemicals can target CSCs of different cancers.

In addition to plants, molecules targeting CSCs have been found in other sources. 3-O-methylfunicone (OMF), a secondary metabolite of the soil fungus *Penicillium pinophilum*, inhibited spheroid cultures of human breast CSCs from the cell line MCF7 (Buommino et al. 2011). Thus, fungal metabolites may act on CSCs.

Vitamins target CSCs. Retinoic acid, a metabolite of vitamin A, is a molecule essential for growth and development; it also induces cell differentiation. All-*trans*

Table. 2 Probable Molecular Targets for Phytochemicals in Cancer Stem Cells (CSCs). (Adapted from Kawasaki et al. 2008; Subramaniam et al. 2010; Li et al. 2011, and the references cited in text)

Targeted process	Molecular targets	Phytochemicals (Examples Of Sources)
Signaling Pathways		
Hedgehog	Gli Smo	Cyclopamine [Corn lily]
		EGCG [Green tea]
		Genistein [Soy]
		Quercetin [Apples, onions]
		Sulforaphane [Broccoli, cabbages]
Wnt	β -catenin	EGCG [Green tea] Sulforaphane [Broccoli, cabbages]
Notch	Notch	Curcumin [Turmeric] Resveratrol [Grapes, peanuts]
Kinases	Protein tyr kinase	Genistein [Soy]
Inflammation	NF κ B IL-6 STAT-3	Curcumin [Turmeric] Parthenolide [Feverfew]
		Resveratrol [Grapes, peanuts]
		Cucurbitacin [Cucumber, pumpkins]
		Resveratrol [Grapes, peanuts]
Drug Resistance		
Efflux	ABCG2	Curcumin [Turmeric] Piperine [Black pepper]
Metabolism	ALDH AMPK Hsp 27 ROS	Curcumin [Turmeric] Sulforaphane [Broccoli, cabbages]
		EGCG [Green tea]
		Quercetin [Apples, onions]
		EGCG [Green tea]
		Sulforaphane [Broccoli, cabbages]
EMT	Slug	Resveratrol [Grapes, peanuts]
Metastasis	MMP-9	EGCG [Green tea]
Apoptosis	Bax	I3C [Broccoli, cabbages]
	Caspase	Resveratrol [Grapes, peanuts]
Autophagy	LC3	Curcumin [Turmeric]
Differentiation	RAR	Retinoic acid [Spinach, carrots]

ABCG-2 ATP-binding cassette transporter 2, *ALDH* aldehyde dehydrogenase, *AMPK* AMP-activated protein kinase, *Bax* B cell lymphoma 2 (Bcl-2) associated X protein is a proapoptotic protein, *β -catenin* beta catenin, a protein in the Wnt signaling pathway, *Caspase* cysteine-dependent aspartate-directed protease are proteinases functional in apoptosis, *EGCG* epigallocatechin gallate, *EMT* Epithelial mesenchymal transition, *Gli* zinc finger transcription factor in Hedgehog signaling pathway, *Hsp 27* heat shock protein 27 is a chaperone protein, *I3C* indole-3-carbinol, *IL-6* interleukin 6, *LC3* microtubule associated protein light chain 3 is a marker for autophagy, *MMP-9* matrix metalloproteinase 9, *NF κ B*, transcription factor nuclear factor kappa B, *Notch* a membrane protein in the notch signaling pathway, *RAR* retinoic acid receptor, *ROS* reactive oxygen species, *STAT 3* signal transducer and activator of transcription 3, *Slug* transcription factor Slug is important for EMT, *Smo* plasma membrane Smoothed in Hedgehog signaling pathway, *Tyr* tyrosine

retinoic acid (ATRA) inhibited human breast CSCs (Ginestier et al. 2009); ATRA stealth liposomes prevented relapse of breast cancer arising from breast CSCs (Li et al. 2011). Gemini vitamin D, a vitamin D analogue, targets human breast CSCs (So et al. 2011). *Gamma*-tocotrienol, a vitamin E constituent, targets human prostate CSCs (Luk et al. 2011).

Some drugs may also target CSCs. The telomerase inhibitor imetelstat inhibited both breast and pancreatic CSCs (Joseph et al. 2010). Statins, including simvastatin and lovastatin, suppressed abnormal human embryonic stem cells and thus may play a role in CSC inhibition (Gauthaman et al. 2009). Probably the most significant drug reported thus far for targeting CSCs is metformin (Hirsch et al. 2009; Vazquez-Martin et al. 2011; Iliopoulos et al. 2011). Metformin is currently being used to treat diabetes. There is a definite future in its application to CSCs (Dowling et al. 2012).

High-throughput screening assays on currently available compounds have been established for embryonic and CSCs. In one human embryonic stem cell screen, retinoic acid and others have been found to modulate Oct4 expression (Desbordes et al. 2008). In a breast CSC screen, salinomycin has been found to target CSCs. This compound, originally isolated from *Streptomyces albus*, is an ionophore and has been added to chicken feed as a coccidiostat to control protozoan parasites in poultry (Gupta et al. 2009b). In another ovarian CSC screen from 1,200 clinically approved drugs, the compound niclosamide has been found to target CSCs (Yo et al. 2012). Originally developed as a molluscicide (for snails that are intermediate hosts of blood flukes, parasites causing schistosomiasis) and anthelmintic (for cestodes or tapeworms), niclosamide has a proton carrier mode of action and is also active against viruses (such as the one that causes severe acute respiratory syndrome, SARS; Jurgeit et al. 2012). In a combined normal and CSC screen, the antipsychotic drug thioridazine has been shown to target only CSCs, whereas salinomycin targets both normal and CSCs (Sachlos et al. 2012). It is expected that more compounds targeting CSCs will be uncovered in future screenings.

A complementary approach is to look for novel sources that target CSCs. Along this line of research, an extract from the marine sponge *Crambe crambe* has been shown to target pancreatic and prostate CSCs (Ottinger et al. 2012). Thus, unusual animal and plant species may yield novel molecules targeting CSCs.

Desirable Properties of Compounds Targeting Cancer Stem Cells

Phytochemicals and other compounds that target CSCs have just been reviewed. Here we discuss desirable properties needed for application to cancer therapy. Topics of concern are antagonistic effects, combination therapy, multiple molecular targets, safety, and bioavailability.

Whereas synergistic effects among phytochemicals are welcome in cancer prevention and therapy (Liu 2004; HemaIswarya and Doble 2006), antagonistic effects are not. We can use curcumin, resveratrol, and EGCG as examples. Curcumin is

synergistic with resveratrol in colon cancer inhibition (Majumdar et al. 2009), but antagonistic with EGCG towards keratinocytes. EGCG enhances keratinocyte differentiation whereas curcumin inhibits its differentiation; curcumin activates apoptosis whereas EGCG does not (Eckert et al. 2006). Potential antagonistic effects among compounds must be analyzed before combination use.

Despite the potential opposing effects between individual phytochemicals, their combinatorial applications have been promoted. It has been suggested that “Specific combinations of phytochemicals may be far more effective in protecting against cancer than isolated compounds” because “combinations of dietary chemopreventive agents can sometimes result in significant activities at concentrations where any single agent is inactive” (De Kok et al. 2008). Thus, combinatorial use of phytochemicals is an attractive approach in targeting CSCs.

Each CSC has multiple molecular targets. Dietary phytochemicals such as curcumin, resveratrol, and EGCG tend to aim at a multitude of cellular targets. Perhaps it is because of these characteristics that definitive mechanisms of action are not available despite decades of research (Francy-Guilford and Pezzuto 2008). This multitarget nature of phytochemicals may be advantageous in targeting CSCs because the multifaceted mode of action may hinder the cell’s ability to develop resistance to the phytochemicals.

How safe are the dietary phytochemicals? Because they can interact with drug transporters and drug metabolizing enzymes (known as phase I and phase II enzymes), the potential toxicity and safety of phytochemicals are major concerns. As an example, they have antioxidant capacities against ROS. However, under special conditions they also exhibit pro-oxidant capacity. Whereas ROS is harmful in general, the removal of too much ROS interferes with body functions, as seen in the warning presented by scientists to the food industry (Finley et al. 2011). With an emphasis on their putative cancer chemopreventive uses, the safety of a group of phytochemicals has been reviewed (Verschoyle et al. 2007). For curcumin, given to cancer patients at 3,600 mg/day for 4 months or 800 mg/day for 3 months, only minor adverse effects were seen. For EGCG, daily oral doses for 4 weeks at 800 mg/day in 40 volunteers caused only minor adverse effects. For resveratrol, a single oral dose at 5 g in 10 volunteers caused only minor adverse effects (Boocock et al. 2007). However, “High consumption of dietary phytochemicals should be considered with caution taking into account their dosage regimes, toxicity, metabolic conversion, transport mechanisms, tissue availability, synergistic interaction with drugs, and interferences with key enzymes, receptors, metabolic pathways, and normal GI microflora” (Priyadarsini and Nagini 2012). More extensive toxicological testing is recommended, if phytochemicals are to be taken as cancer chemopreventive agents on a long-term basis by healthy individuals.

How bioavailable are the dietary phytochemicals? Bioavailability may differ, depending on conditions varying from pure compounds and plant extracts, to mixtures with other food components (EGCG vs. green tea extract, genistein vs. soymilk or tofu, quercetin vs. apple cider or apples; Manach et al. 2005). For phytochemicals such as plant polyphenols, the effects *in vivo*, although significant, are more limited than those observed *in vitro* (Williamson and Manach 2005). However, it is possible to design appropriate interactions that affect the bioavailability of phytochemicals *in vivo* (Scholz

and Williamson 2007), such as enhancement of curcumin efficacy with piperine, as previously mentioned. We have used oral gavage plus starvation (an empty stomach) to enhance curcumin uptake in mice (Chan et al. 1998; Adapala and Chan 2008) so as to demonstrate its *in vivo* inhibition of the inflammatory iNOS in the murine liver (beneficial outcome) and the exacerbation of murine visceral leishmaniasis, a protozoan parasitic disease (detrimental outcome). The last report recommends caution with regard to the potential clinical use of curcumin: it may re-activate latent infections.

A second look at bioavailability relates to the concept of hormesis. The effect of curcumin has been described as an example of xeno-hormesis (Salvioli et al. 2007). The concept of hormesis suggests that the fundamental nature of the dose–response curve to be neither linear nor threshold, but rather U-shaped or J-shaped. This shows a low dose stimulatory response known as the hormetic effect, representing over-compensation in response to disruptions in homeostasis (Calabrese and Baldwin 2001). For phytochemicals among an estimated 10,000 secondary products (natural pesticides), it has been proposed that human ancestors evolved a generalized defense mechanism against low levels of phytochemicals to enable their consumption of many different plant species containing variable levels of natural pesticides (carcinogens) without subsequent ill health. Traces of phytochemicals found in fruits and vegetables may potentiate the immune system and help to protect against cancer (Trewavas and Stewart 2003). A biphasic dose response is observed. At high concentrations, phytochemicals can be toxic, whereas subtoxic doses may induce adaptive stress responses. There is the activation of signaling pathways that results in increased expression of genes encoding cytoprotective proteins. It is suggested that hormetic mechanisms of action may underlie many of the health benefits of phytochemicals (Mattson 2008). These benefits would include their action against CSCs.

Implementation of the Phytochemical Approach Towards Chemoprevention

Among all the compounds undergoing clinical trials, the success rate of anticancer drugs is less than 5%, the lowest as compared to other drugs for cardiovascular, central nervous system, and infectious diseases; and it takes \$ 2 billion to bring a drug to market (Bhattacharjee 2012). Examples of anticancer drugs from plants are taxol and vinblastine. Plants are a rich source of drugs. “Only a small fraction of the immense diversity of plant metabolism has been explored for the production of new medicines and other products important to human well-being” (De Luca et al. 2012). Therefore, new phytochemicals targeting CSCs are expected in the future.

In the meantime, currently available phytochemicals need more definitive studies, because an evidence-based approach is favored (Higdon 2007). “Facts and fiction” of each phytochemical must be differentiated (Espín et al. 2007). A phytochemical may act in concert with conventional drugs, as visualized by the following metaphor. When used in low concentrations, the phytochemical causes multiple wounds without killing the CSC. Nevertheless, the treatment increases the CSC’s

vulnerability to the magic bullet represented by the chemotherapeutic drug (Russo 2007). In this sense, dietary phytochemicals may serve as adjuvant anticancer agents with practically minimal or no side effects.

There is an urgent need to bridge the gap between apparent *in vitro* efficacy and clinical use of dietary phytochemicals (Manson et al. 2007). Biomarkers and decisions on dosages for clinical trials still have to be worked out (Scott et al. 2009). As an example, transcription analyses (microarrays of gene expression) of peripheral blood mononuclear cells (PMBCs) from dietary intervention studies “have not resulted yet in clear confirmation of candidate genes related to disease risk” (De Mello et al. 2012).

What is the current status of dietary phytochemicals for cancer chemoprevention? The focus lies in the identification of molecular mechanisms and cellular targets. The “concept of combination chemoprevention by multiple agents or by the consumption of ‘whole foods’ has become an increasingly attractive area of study” (Mehta et al. 2010).

Concerning “whole foods” consumption, the current US dietary guidelines have been illustrated as MyPlate, with advice to cover half the plate with fruits and vegetables (Willett and Ludwig 2011). From Europe, there is Mediterranean Diet Pyramid Today, with a plant-based core of the dietary pattern for consuming fruits and vegetables everyday (Bach-Faig et al. 2011).

How effective are fruits and vegetables for cancer chemoprevention? The early case-control studies tout the reduction in cancer risk, but the more recent prospective cohort studies show only a weak association. For example, a pooled analysis of 14 cohort studies of pancreatic cancer concludes that “fruit and vegetable intake during adulthood is not associated with a reduced pancreatic cancer risk” (Koushik et al. 2012). Therefore, this is “turmoil in the produce section” but: “A very weak or undetectable association between fruits and vegetables and risk of cancer does not exclude the possibility that one or a small group of fruits or vegetables, or a specific substance in some of these foods, has an important protective effect” (Willett 2010). This latter point has been well proven by the catalogue of dietary phytochemicals that can target CSCs covered in this review. Furthermore, “although the evidence for benefits of fruits and vegetables against cancer was waning, data supporting benefits for cardiovascular disease were accumulating” (Willett 2010).

Perspectives

In September 2011, the United Nations General Assembly convened a High Level Meeting on Non-communicable Diseases (NCDs) because the four main NCDs—cardiovascular disease, cancer, chronic lung diseases, and diabetes—kill three in five people worldwide, and cause great socioeconomic harm within all countries, particularly developing nations. In September 2012, *Science* magazine published a special section including a cover photo on the topic of disease prevention (Ash et al. 2012). One contributed article titled: “Can Noncommunicable Diseases Be

Prevented?” suggested “effective approaches for large-scale NCD prevention include,” among these, “increasing the consumption of fresh fruits and vegetables” (Ezzati and Riboli 2012).

When is the best time to start eating fruits and vegetables? First Lady Michelle Obama started a campaign against childhood obesity (Willett and Ludwig 2011). It has been suggested that “research should focus more sharply on specific fruits and vegetables and their constituents and on earlier periods of life” (Willett 2010). We have already mentioned genistein’s effect on fetal DNA methylation. Thus, here comes “the maternal womb: a novel target for cancer prevention” (Simmen and Simmen 2011). Dietary interventions prior to and during pregnancy may confer cancer chemoprevention. The first sentence in a discussion on the “myth or reality” aspects of dietary molecules begins with the 400 BC statement of Hippocrates: “Let thy food be thy medicine and thy medicine be thy food” (Neergheen-Bhujun et al. 2012).

We end this chapter with a comment on cell differentiation and the CSC. In October 2012 the Nobel Prize in Physiology or Medicine was announced for the discovery of cell reprogramming (Abbott 2012). In 1962 John Gurdon published his work on the plasticity of a differentiated nucleus from an amphibian: the nucleus from a tadpole’s intestinal cell replaced the egg nucleus and generated a frog. In 2006 Shinya Yamanaka published his work on the reprogramming of a differentiated cell type from a mouse: the mouse fibroblast became an embryonic stem cell by adding just four genes. The latter work is transferable to humans and the induced pluripotent stem (iPS) cells will be important for future clinical applications. The differentiated state of a cell is dynamic. A CSC plays its essential role in maintaining the particular tumor. If dietary phytochemicals, or specific fruits and vegetables, are available to destroy this CSC, or reverse its stemness characteristics, or induce its cell differentiation, there will be chemoprevention of cancer.

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Basic and Translational Research on Dietary Phytochemicals and Cancer Prevention

Ashraful Hoque and Xiao-Chun Xu

Abstract Natural or chemically modified dietary phytochemicals are particularly favored for cancer prevention because they can reverse, suppress, or prevent the initial phase of carcinogenesis or the neoplastic cell progression to cancer mass, and therefore, eliminate premalignant and malignant cell populations either in general or in high-risk populations. Screening and identifying dietary phytochemicals for chemoprevention requires in vitro studies of cell lines and animal models before translation into clinical trials. Understanding the molecular pathways that dietary phytochemicals modify could help in designing successful cancer prevention clinical trials. To date, a number of chemoprevention clinical trials have been conducted with limited success. One of the reasons for unsuccessful clinical trials was the lack of a clear molecular target against a particular cancer type. High-throughput screening of phytochemicals to induce premalignant and malignant cell apoptosis by targeting multiple gene pathways should be assessed for future prevention of human cancers.

Keywords Dietary phytochemicals · Cancer prevention · Apoptosis · Gene expression · COX-2 · EGCG · Curcumin · Guggulsterone · Genistein · Resveratrol · Lycopene

Introduction

As many as 10,000 phytochemicals, a number of which are available through dietary supplements or consumption of whole foods, may be useful in treating or preventing various diseases, including cancer (Surh 2003; Aggarwal and Shishodia 2006; Khan et al. 2008; Stan et al. 2008; Lee et al. 2011b; Tan et al. 2011; Singh et al. 2011). The development of cancer is caused by multiple genetic alterations of normal cells (such as the loss, mutation, or epigenetic modification of DNA) that alter normal cell homeostasis by promoting indefinite cell proliferation while inhibiting cell death (Mitchell et al. 2009). Epidemiological studies have demon-

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strated that a large number of cancers are caused by unhealthy lifestyles, such as tobacco smoking, alcohol consumption, and unbalanced diets (e.g., eating fats or red meat). Dietary factors alone may contribute to more than 30% of human cancers (Surh 2003). In contrast, consuming fruits and vegetables could reduce the incidence of cancer because they may contain biologically active phytochemicals that block or inhibit tumorigenesis by targeting the molecular signaling transduction pathways of tumor cells and thus prevent or delay tumor development (Surh 2003; Aggarwal and Shishodia 2006; Khan et al. 2008; Stan et al. 2008; Lee et al. 2011b; Singh et al. 2011). This chapter updates the evidence on the usefulness of dietary phytochemicals in preventing cancer.

Dietary Phytochemicals as Chemoprevention Agents to Suppress Tumorigenesis

Inhibition of Tumor Initiation

Hanahan and Weinberg (2000) summarized six essential hallmarks of cancer cells: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. During tumorigenesis, cells lose normal homeostasis control and transform into premalignant or malignant phenotypes. These alterations are gained through the mutation or loss of cell growth-critical genes or epigenetic changes in genomic DNA to silence tumor suppressor genes or to activate oncogenes (Hanahan and Weinberg 2000; Mitchell et al. 2009).

However, phytochemicals and other agents can suppress, reverse, or delay the initiation of one or more of these cancer cell hallmarks (Surh 2003; Aggarwal and Shishodia 2006; Khan et al. 2008; Stan et al. 2008; Lee et al. 2011b; Tan et al. 2011; Singh et al. 2011). Research over the last three decades supports the premise that a diet rich in fruits and vegetables can protect against various types of cancers (Khan et al. 2008; Tan et al. 2011). For example, (-)-epigallocatechin-3-gallate (EGCG) and other polyphenols suppressed chemical-induced carcinogenesis in the lung (Xu et al. 1992; Katiyar et al. 1993; Cao et al. 1996; Chung et al. 1998), forestomach (Katiyar et al. 1993), liver (Cao et al. 1996), esophagus (Wang et al. 1995; Li et al. 2002b; Hou et al. 2005), and colon (Yamane et al. 1991; Huang et al. 1994; Peng et al. 2006; Conney 2003) of rodent models. Identifying the targets of phytochemicals is the key for successfully suppressing tumorigenesis in *in vitro* experiments, animal models, and clinical trials.

Different cancers have different tumor initiation processes, which are relatively easy to monitor or control in animal experiments [such as nicotine-derived nitrosamine ketone- or 4-nitroquinoline 1-oxide-induced lung (Yano et al. 1994; Zheng and Takano 2011) or head and neck (Tang et al. 2004)], and esophageal cancers (Wang et al. 1995; Li et al. 2002b; Tang et al. 2004)] but not in clinical trials because invasive (i.e., clinically detectable) tumors can take decades to form. Previous

reviews have listed a number of phytochemicals available for in vitro experiments, animal studies, and even clinical chemoprevention trials against different human malignancies (Prasain and Barnes 2007; Patel et al. 2007; Khan et al. 2008; Lee et al. 2011b) or provided a list of potential molecular targets of different phytochemicals (Aggarwal and Shishodia 2006; Lee et al. 2011b).

Some phytochemicals (such as ellagic acid, sulphoraphane, and flavonoids) are tumor-blocking agents, but most are tumor-suppressive agents (such as EGCG, curcumin, β -carotene, genistein, resveratrol, and ginerol) or both (Aggarwal and Shishodia 2006; Johnson 2007; Patel et al. 2007; Khan et al. 2008). A number of carcinogens used in animal experiments need to be activated by cytochrome P450 or detoxified by phase II enzymes in cells (Wattenberg 1985; Surh 2003; Froyen et al. 2009). Some phytochemicals can either inhibit cytochrome P450 or activate phase II enzymes to block the tumor initiation process by antagonizing carcinogen-induced damage to genomic DNA and thus preventing or delaying cancer development (Surh 2003).

Inhibition of Tumor Progression

Because a clear tumor initiation stage cannot be defined for most human cancers, the use of dietary phytochemicals to suppress tumor promotion or progression could make sense. There is no consensus as to whether it is better to use agents to prevent or delay premalignant lesions from progressing to invasive cancer or to use agents that induce apoptosis of malignant cells. For studies on preventing cancer incidence, animal models (such as chemical-induced tumor or transgenic mouse tumor models) are usually used with pharmacological doses of phytochemicals. For studies on inducing cancer cell apoptosis, premalignant and malignant cell lines are mainly used to test the effectiveness of pharmacological levels of dietary phytochemicals in vitro and then animal experiments or human clinical trials are conducted.

Induction of Premalignant and Malignant Cells to Undergo Apoptosis

Apoptosis is a multistep, multipathway-triggered programmed cell death that involves expression of a number of genes and their activation (Hail and Lotan 2009). Mechanistically, there are two pathways to trigger cell apoptosis: the mitochondria-initiated caspase-9 pathway and the death-receptor-initiated nonmitochondrial caspase-8 pathway (Israels and Israels 1999). These two pathways do crosstalk, and some phytochemicals can activate both pathways (Israels and Israels 1999; Ye et al. 2012; Guan et al. 2013). Thus, inducing apoptosis could be an effective approach to eliminate premalignant and malignant cells from the human body. Various dietary phytochemicals target multiple gene pathways and induce the apoptosis of premalignant and malignant cells, resulting in the suppression and prevention

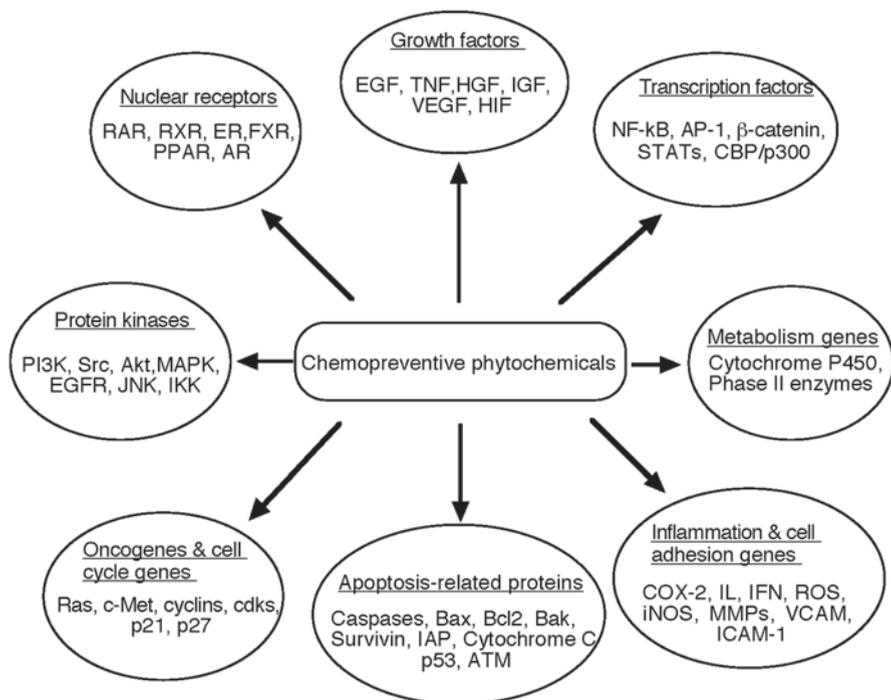


Fig. 1 Potential molecular targets of chemopreventive phytochemicals, an uncompleted list (modified from Aggarwal and Shishodia 2006; Khan et al. 2008)

of tumorigenesis or tumor progression (Aggarwal and Shishodia 2006; Hail and Lotan 2009). A great number of phytochemicals, such as EGCG, curcumin, and guggulsterone, have the ability to induce tumor cells to apoptosis, sparing normal cells (Conney 2003; Urizar and Moore 2003; Aggarwal and Shishodia 2006; Ye et al. 2012).

Phytochemical-Modulated Molecular Pathways in Induction of Transformed Cell Apoptosis

Various phytochemicals regulate gene pathways to induce premalignant and malignant cells to undergo apoptosis (Surh 2003; Aggarwal and Shishodia 2006; Khan et al. 2008; Stan et al. 2008; Lee et al. 2011b; Tan et al. 2011; Singh et al. 2011). Our recent study showed that guggulsterone was able to increase RAR- β_2 expression and inhibit COX-2 expression to activate caspases-8 and -9 to induce apoptosis of esophageal cancer cells (Guan et al. 2013). Figure 1 summarizes some known molecular targets of currently available chemopreventive phytochemicals, which are further discussed in other chapters. In this chapter, we focus

primarily on the RAR- β_2 -led gene pathway using different phytochemicals to prevent human carcinogenesis.

Several lines of evidence have supported the association between loss of RAR- β_2 expression and increased carcinogenesis (Soprano et al. 2004; Xu 2007; Mongan and Gudas 2007). Induction of RAR- β_2 expression in esophageal cancer cells suppressed tumor cell growth and colony formation and induced apoptosis in vitro and in nude mouse xenografts (Xu et al. 1999; Li et al. 2002a). In contrast, esophageal, lung, and breast cancer cell lines that do not express RAR- β_2 have been shown to be resistant to retinoid treatment (Houle et al. 1991; Xu et al. 1999; Ren et al. 2005). Furthermore, our studies linked RAR- β_2 expression with downregulation of COX-2 expression in vitro and ex vivo (Li et al. 2002a; Song et al. 2005, 2009).

COX-2, a key enzyme in the conversion of arachidonic acid to prostaglandins, is frequently overexpressed in many different types of human cancer (Xu 2002; Menter and Dubois 2012). The tobacco carcinogen BPDE and tumor-promoting bile acids induced COX-2 expression in head and neck and esophageal cancer cells (Kelley et al. 1997; Zhang et al. 1998; Li et al. 2002a; Song et al. 2005, 2009). Restoration of RAR- β_2 expression suppressed COX-2 expression in esophageal cancer cells, and 13-*cis* retinoic acid was able to inhibit COX-2 expression in patients with oral premalignant lesions (Song et al. 2009). In addition, RAR- β_2 suppression of COX-2 expression is known to occur through the EGFR/Erk1/2/AP-1 molecular pathway (i.e., restoration of RAR- β_2 expression in esophageal cancer cells suppressed COX-2 expression), whereas induction of COX-2 by BPDE was mediated through inhibition of RAR- β_2 and, consequently, induction of Erk1/2 phosphorylation and AP-1 expression (Xu 2007). In contrast, esophageal cancer cells that do not express RAR- β_2 did not respond to BPDE for induction of COX-2. Another study showed that a high-fat diet inhibited RAR- β mRNA, but increased COX-2 mRNA expression and aberrant crypt foci formation in rat colon, whereas vitamin A was able to prevent these alterations (Delage et al. 2005). Thus, these studies suggest that these genes may form a novel molecular pathway that involves RAR- β_2 -suppressed EGFR/p-Erk1/2/AP-1/COX2 in human cancers (Xu 2007, 2009).

A previous study showed that EGCG was able to induce RAR- β_2 expression (Fang et al. 2003). Our data showed that curcumin, EGCG, and lovastatin or their combination were able to suppress esophageal cancer cell growth in vitro and in nude mouse xenografts and that these agents inhibited Erk1/2 phosphorylation, c-Jun, and COX-2 expression (Ye et al. 2012). Guggulsterone was able to suppress esophageal cancer cell viability; induce apoptosis by activation of caspases-8, -9, and -3 in tumor cells in vitro; and reduced tumor growth in nude mouse xenografts (Guan et al. 2013). In addition, various epidemiology studies showed that suppression of COX-2 activity by regular use of aspirin was associated with a consistently reduced risk of various cancers, such as colorectal and esophageal cancers (Xu 2002; Chan and Detering 2012; Thun et al. 2012). Thus, targeting of the RAR- β_2 -led gene pathway could effectively prevent a number of human cancers in various organs.

Phytochemicals as Chemopreventive Agents to Control Human Cancer *In Vitro* and in Animal Models

To date, both human and animal studies have demonstrated that most dietary phytochemicals can modulate the expression of cell growth-critical genes *in vitro* and *in vivo* in favor of cancer risk prevention (Surh 2003; Aggarwal and Shishodia 2006; Khan et al. 2008; Stan et al. 2008; Lee et al. 2011b; Tan et al. 2011; Singh et al. 2011). The following sections describe several phytochemicals that have been investigated for their efficacy in preventing cancer.

Tea Extracts: EGCG and Polyphenols

EGCG and polyphenols from green tea extract are the most extensively studied dietary phytochemicals for cancer chemoprevention (Conney 2003; Brahma et al. 2011; Singh et al. 2011; Yang et al. 2011b; Kanwar et al. 2012). EGCG and its analogues possess antioxidative activities and modulate xenobiotic metabolic enzymes to prevent oxidative damage in healthy cells (Glei and Pool-Zobel 2006; Lambert and Elias 2010), and they also inhibit tumor promotion and progression (Conney 2003; Yang and Wang 2011). *In vitro*, EGCG was able to enhance tumor cell apoptosis, suppress proliferation and invasion, and inhibit angiogenesis in a great number of cancer cell lines (Conney 2003; Lambert and Yang 2003; Yang et al. 2011a; Singh et al. 2011; Kanwar et al. 2012). EGCG has been shown to have chemopreventive activity by suppressing carcinogenesis in many organ sites through inhibition of tumorigenesis during the initiation, promotion, and progression stages (Yamane et al. 1991; Xu et al. 1992; Katiyar et al. 1993; Wang et al. 1995; Cao et al. 1996; Chung et al. 1998; Li et al. 2002b; Conney 2003; Hou et al. 2005; Peng et al. 2006; Kanwar et al. 2012). However, the mechanisms of the cancer chemopreventive activity of EGCG are not completely characterized and many features remain to be elucidated. Molecularly, EGCG possesses antioxidant/pro-oxidant activity, inhibits growth factor signaling (such as EGFR, PI3K, and Erk1/2), and inhibits the activity of secreted MMP2 and MMP9 and AP-1 and STAT3 activities (Conney 2003; Lambert and Yang 2003; Vayalil and Katiyar 2004; Yang et al. 2011a; Singh et al. 2011; Kanwar et al. 2012). EGCG reactivated silenced tumor suppressor genes (such as Cip1/p21, p16INK4a, and RAR- β_2) by reducing DNA methylation and increasing histone acetylation (Fang et al. 2003; Conney 2003; Nandakumar et al. 2011; Yang and Wang 2011). Previous studies showed that EGCG was able to induce RAR- β_2 but inhibited COX-2 expression in esophageal cancer cells (Fang et al. 2003; Ye et al. 2012). EGCG can also stimulate telomere fragmentation by inhibiting telomerase activity (Meeran et al. 2011; Singh et al. 2011). Green tea provides overall health benefits by not only preventing cancer but also in cardiovascular and metabolic diseases (Serafini et al. 2011). Further investigation and identification of the molecular targets of EGCG would greatly facilitate understanding of the mechanisms underlying its anticancer and cancer preventive activities.

Turmeric Extract: Curcumin

Curcumin is a polyphenolic antioxidant with low toxicity that is derived from dietary spices frequently used in Indian food (Aggarwal et al. 2003; Kuttan et al. 2007). Curcumin comprises 2–8% of most turmeric preparations (Tayyem et al. 2006; Sharma et al. 2007) and exhibits anti-inflammatory, anticarcinogenic, antiproliferative, antiangiogenic, and antioxidant properties in various cancer cell models and has been shown to inhibit different cancers at the initiation, promotion, and progression stages in animal models (Aggarwal et al. 2003; Kuttan et al. 2007). Curcumin showed the ability to suppress growth and induce apoptosis in many types of cancer cells in vitro (Aggarwal et al. 2003; Kuttan et al. 2007). Recently, its molecular mechanisms of action have been extensively investigated and its efficacy appears to be related to the induction of glutathione and glutathione-S-transferase activity, inhibition of lipid peroxidation and arachidonic acid metabolism, and suppression of oxidative DNA adduct formation (Aggarwal et al. 2003; Joe et al. 2004; Lin 2007; Kuttan et al. 2007). Curcumin is a potent inhibitor of protein kinase C, EGFR, and I κ B (Aggarwal et al. 2003; Lin 2004). Curcumin can inhibit the activation of NF- κ B and the expression of c-Jun, c-Fos, c-Myc, Erk1/2, COX-2, PI3K, Akt, CDKs, and iNOS (Maheshwari et al. 2006; Lin 2007; Ye et al. 2012). Curcumin was also able to suppress cigarette smoke-induced NF- κ B activation and COX-2 expression in head and neck squamous cell carcinoma and non-small-cell lung cancer cells (Shishodia et al. 2003; Aggarwal et al. 2004). In esophageal cancer, dietary curcumin can inhibit chemically induced esophageal carcinogenesis in mice and rats (Huang et al. 1994; Ushida et al. 2000). However, its low bioavailability could be an issue in clinical use because of its extreme lipophilicity and instability (Kidd 2009; Bar-Sela et al. 2010). Thus, development of different formulations (such as nanoparticles, liposomes, or microemulsions) and biosynthesis of more stable and absorbable curcumins could improve its bioavailability for human cancer chemoprevention (Ohuri et al. 2006; Shahani et al. 2010). Curcumin has the potential to treat a wide variety of other illnesses as well, such as inflammatory disease, diabetes, cardiovascular disease, arthritis, and Alzheimer's disease (Pari et al. 2008; Mishra and Palanivelu 2008).

Guggul Extract: Guggulsterone

Guggul, a herbal extract from resin of the *Commiphora mukul* tree, is one of many widely used ancient Ayurvedic drugs that exhibit activity against different diseases, including cancer, when taken orally (Urizar and Moore 2003; Shah et al. 2012; El-Mekkawy et al. 2012). Its active constituents, the Z- and E-guggulsterones, have a high degree of human bioactivity and biological activities through binding to nuclear receptors and modulation of the expression levels of genes involved in carcinogenic activities (Urizar and Moore 2003; Shishodia et al. 2008). Guggulsterone can modulate a number of molecular targets, including growth factors and their

transcription factors, cytokines, enzymes, and apoptosis-related genes (Urizar and Moore 2003; Shishodia et al. 2007, 2008).

Guggulsterone was recently described as a farnesoid X receptor (FXR) antagonist (Urizar et al. 2002; Shishodia et al. 2007, 2008). FXR is a member of the nuclear hormone receptor superfamily and uses bile acid as its ligand (Makishima et al. 1999; Parks et al. 1999). Guggulsterone can suppress the activation of NF- κ B and ERK1/2 and the expression of COX-2 and AP-1 (Shishodia et al. 2007, 2008). Guggul-inhibited COX-2 activity could explain at least in part the anti-inflammatory activity and anticancer effects of guggulsterone in animal experiments (Urizar and Moore 2003; Shishodia et al. 2007, 2008). Guggulsterone sensitizes hepatoma cells to TRAIL-induced apoptosis in hepatocellular carcinoma cells (Moon et al. 2011) and prevented activation of the smokeless tobacco-induced PI3K/Akt pathway in head and neck cancer cells (Macha et al. 2011). Guggulsterone also inhibited angiogenesis by blocking STAT3 and VEGF expression in colon cancer cells and constitutive and inducible STAT3 activation (Ahn et al. 2008; Kim et al. 2008). Guggulsterone was able to induce apoptosis of the highly FXR-expressing Barrett's esophageal and esophageal cancer cells in vitro (De Gottardi et al. 2006; Guan et al. 2013).

FXR is expressed at high levels in the liver and intestine to regulate bile acid and lipid metabolism by suppressing cholesterol 7 α -hydroxylase, the rate-limiting enzyme in bile acid synthesis from cholesterol (Makishima et al. 1999; Parks et al. 1999). FXR is also involved in lipid and glucose metabolism in the human body and plays a role in growth regulation, apoptosis, and carcinogenesis (Kuipers et al. 2007). Studies have established both positive and reciprocal correlations between FXR expression and cancer (Swales et al. 2006; Kuipers et al. 2007; Wang et al. 2008; Modica et al. 2008; Maran et al. 2009; Journe et al. 2009; Das et al. 2009). FXR protects liver cells from apoptosis induced by serum deprivation in vitro and fasting in vivo (Wang et al. 2008). FXR has also been shown to protect against intestinal and liver tumorigenesis (Modica et al. 2008; Maran et al. 2009).

The expression and functions of FXR in other organ systems show a different picture. For example, FXR was overexpressed in pancreatic cancer and associated with lymph node metastasis in ex vivo and FXR expression promoted tumor cell migration and invasion (Lee et al. 2011a). The FXR agonist and chenodeoxycholic acid significantly increased endothelial cell motility and tube formation, and increased cell motility was associated with prominent increases in focal adhesion that was inhibited by FXR and MMP-9 short interfering RNA (Das et al. 2009). In esophageal cancer, FXR expression was increased in esophagitis, Barrett esophagus, and adenocarcinoma compared with FXR expression in normal mucosa (De Gottardi et al. 2006). In vitro treatment with guggulsterone was associated with a significant increase in apoptosis and caspase-3 activity in Barrett esophagus-derived cells (De Gottardi et al. 2006). Bile acid-stimulated FXR expression was shown to enhance the immune response in Barrett esophagus (Capello et al. 2008). Guggulsterone suppressed tumor formation and growth in nude mouse xenografts of esophageal cancer cell lines, similar to the effects of FXR shRNA (Guan et al. 2013).

Soybean Compound: Genistein

Genistein, one of the most active natural flavonoids, exerts antioxidative, antiproliferative, anticancer, and other biological effects (Sarkar and Li 2003). Genistein can regulate the AR/Akt/PTEN axis as one of the molecular mechanisms for inhibition of cell proliferation and induction of apoptosis in prostate cancer prevention and treatment (Wang et al. 2009). Genistein was also able to suppress prostaglandin synthesis and activity in human prostate cancer cells and in prostate cancer patients (Swami et al. 2009) and inhibited prostate cancer in animals (Harper et al. 2009). Moreover, it modulated the expression of genes related to the control of cell cycle and apoptosis (Sarkar and Li 2003). Genistein can also inhibit activation of NF- κ B and Akt, both of which are known to maintain a homeostatic balance between cell survival and apoptosis (Sarkar and Li 2003). Genistein can compete with 17 β -estradiol for estrogen receptor binding because of its structural similarity, resulting in some agonistic or antagonist activity (Sarkar et al. 2006). Thus, genistein has been used mostly to prevent breast and prostate cancers (Barnes et al. 1995). Genistein inhibited both constitutive and EGF-stimulated invasion in estrogen receptor-negative human breast carcinoma cell lines (Shao et al. 1998, b) and suppressed mammary gland tumors in rats (Lamartiniere et al. 1995). To date, more than 30 clinical trials of genistein in various diseases have been conducted to evaluate its clinical efficacy (Yang et al. 2012). Many animal and human pharmacokinetic studies have shown that the most challenging issue for developing genistein as a chemoprevention agent is its low oral bioavailability (Messing et al. 2012; Yang et al. 2012; Tang et al. 2011).

Grape Seed Extract and Resveratrol

Grape seed extract contains a high concentration of vitamin E, flavonoids, linoleic acid, and proanthocyanidin and has antioxidant activity (Vanden Berghe 2012). Dietary feeding of grape seed extract prevents intestinal tumorigenesis in mice (Velmurugan et al. 2010, b). Dietary grape seed proanthocyanidins inhibit UVB-induced COX-2 expression and other inflammatory mediators in UVB-exposed skin and skin tumors in SKH-1 hairless mice (Sharma and Katiyar 2010). Polyphenolic extracts from seedless and seeded Indian grapes had antitumor effects in vitro, showing that they are suitable for cancer chemoprevention (Lee and Lee 2006; Ramchandani et al. 2008). Grape seed extract inhibited prostate tumor growth and progression in TRAMP mice (Raina et al. 2007).

Resveratrol is found in the skin of red grapes and in other fruits and has shown natural antioxidant activities against various human diseases, such as cardiovascular disorders and cancer, and possesses anti-inflammatory activity and positively regulates glucose level and adipose tissue metabolism (Aziz et al. 2003; Bishayee 2009). In animal models, resveratrol suppressed tumor initiation, promotion, and progression and modulated signal transduction pathways that control cell division

and growth, apoptosis, inflammation, angiogenesis, and metastasis (Aziz et al. 2003; Bishayee 2009). Resveratrol supplementation can also prevent metaplasia initiation and carcinogenic progression to esophageal adenocarcinoma (Woodall et al. 2009). Molecularly, resveratrol inhibits the activity of NF- κ B, AP-1, COX-2, MMP-9, MAPKs, iNOS, Pi3K, and STAT-1 proteins (Kundu and Surh 2004; Kundu et al. 2006; She et al. 2003). Resveratrol induced apoptosis by upregulating the expression of Bax, Bak, Bim, p53, and TRAIL but downregulated expression of Bcl-2, Bcl-XL, Mcl-1, and survivin (Pohland et al. 2006; Shankar et al. 2007). The chemopreventive potential of resveratrol in mouse skin tumors was through regulation of cytochrome *c* release from the mitochondria, caspase activation, and PI3K/AKT signaling pathways (Roy et al. 2009). However, its poor oral bioavailability limits its potential clinical application. Pterostilbene, the natural dimethylated analogue of resveratrol, has greater bioavailability with similar bioactivity (Wang et al. 2012).

Tomato Extract: Lycopene

Lycopene is a bright red carotene and carotenoid pigment and phytochemical found in tomatoes and other red fruits and vegetables, such as red carrots, red bell peppers, watermelons, and papayas (Rao and Agarwal 2000; Bhuvanewari and Nagini 2005; Pinela et al. 2012). Although lycopene is chemically a carotene, it has no vitamin A activity (Rao and Agarwal 2000). Epidemiologic studies showed that increasing serum concentrations of lycopene were negatively associated with cervical intraepithelial neoplasia grades 1 and 3 and cancer in low-income Brazilian women (Tomita et al. 2010) and that higher circulating concentrations of lycopene were associated with a lower risk of overall cancer incidence in middle-aged Finnish men (Karppi et al. 2009). Other studies showed evidence of lycopene's benefit for cancers of the lung, stomach, and prostate gland (Rao and Agarwal 2000; Bhuvanewari and Nagini 2005).

A great number of studies have analyzed the anticancer properties of lycopene, although the results have been primarily inconclusive. For example, lycopene has beneficial effects in the management of some premalignant lesions in the oral cavity, such as oral submucous fibrosis and oral leukoplakia, and thus may be useful in preventing oral cancer (Lu et al. 2011). Lycopene suppressed the growth of prostate cancer cells as well (Yang et al. 2011a). A lycopene chemoprevention study of prostate cancer in the TRAMP model showed that the incidence of prostate cancer was significantly lower in mice fed lycopene beadlets than in the control group, whereas the difference between the mice fed tomato paste was not statistically significant (Konijeti et al. 2010). Dietary lycopene and tomato extract supplementations inhibit nonalcoholic steatohepatitis-promoted hepatocarcinogenesis in rats (Wang et al. 2010). Lycopene differentially regulated phase I and II enzymes in dimethylbenz[*a*]anthracene-induced breast cancer cells (Wang and Leung (2010). Lycopene can modulate the expression of various growth factors and growth factor receptors, antioxidant response elements, MAPKs, NF- κ B, AP-1, COX-2, and cyto-

kines (Sengupta et al. 2006; Liu et al. 2006; Huang et al. 2007; Palozza et al. 2011). Lycopene supplementation decreased insulinlike growth factor-I levels in colon cancer patients (Walfisch et al. 2007) but induced circulating insulinlike growth factor binding protein-1 and -2 concentrations in persons at greater risk of colorectal cancer (Vrieling et al. 2007).

The absorption of lycopene, a highly lipid-soluble chemical, can be improved in the presence of a small but essential amount of oil or fat. Monounsaturated oils (such as olive oil or canola oil) are most desirable because they do not increase the risk of atherosclerosis, coronary heart disease, or nutritionally linked cancers (Weisburger 1998). However, there is no credible evidence to support an association between lycopene intake and a reduced risk of prostate, lung, colorectal, gastric, breast, ovarian, endometrial, or pancreatic cancer (Kavanaugh et al. 2007; Kristal et al. 2011; van Breemen et al. 2011; Ilic and Misso 2012). Adding lycopene to other dietary phytochemicals could increase their antitumor activities (Canene-Adams et al. 2007; Shukla and George 2011).

Llyphilized Berries

Multiple types of berry, such as blackberry, red raspberry, strawberry, blueberry, and wolfberry, can prevent carcinogen (such as *N*-nitrosomethylbenzylamine)-induced esophageal cancer in animal models (Stoner et al. 2007, 2008). The berries contain high levels of flavonoids (anthocyanins, flavonols, and flavanols), tannins (proanthocyanidins), hydrolyzable tannins (ellagitannins and gallotannins), stilbenoids, and phenolic acids (Seeram 2006, 2008). Blueberries and cranberries contain predominantly proanthocyanidins, whereas blackberries, black raspberries, red raspberries, and strawberries contain predominantly ellagitannins (Cerde et al. 2004; Seeram 2006, 2008). Among berry phenolics, the anthocyanins are the best studied. Anthocyanins have antioxidant, anticancer, anti-inflammatory, and other biological activities (Cerde et al. 2004; Seeram 2008, 2008, 2009). The freeze-dried berries effectively suppressed both the tumor initiation and promotion/progression stages (Stoner et al. 2007, 2008, 2010). For instance, freeze-dried black raspberries were able to suppress the tumorigenic phenotype in human oral squamous cell carcinoma cells (Han et al. 2005; Rodrigo et al. 2006). Dietary black raspberry powder inhibited NMBA-induced tumor development in the rat esophagus by inhibiting the formation of DNA adducts and reducing the proliferation rate of preneoplastic cells (Kresty et al. 2001; Chen et al. 2006). Dietary anthocyanin-rich tart cherry extract also inhibited intestinal tumorigenesis in *ApcMin*⁺ mice fed suboptimal levels of sulindac (Bobe et al. 2006). Methanol extracts of freeze-dried strawberries and black raspberries inhibited benzo[*a*]pyrene-induced transformation of Syrian hamster embryo cells *in vitro* (Xue et al. 2001).

These berries modulate the expression of cell growth, apoptosis, inflammation, and angiogenesis-related genes, such as AP-1, NF- κ B, COX-2, iNOS, VEGF, inducible nitric oxide synthase, and MMPs (Kresty et al. 2001; Xue et al. 2001;

Casto et al. 2002; Cerda et al. 2004; Chen et al. 2006, b; Lu et al. 2006; Rodrigo et al. 2006; Huang et al. 2002, 2007; Neto 2007; Stoner et al. 2007, 2010). In addition, these berries have been shown to reduce the levels of the serum cytokines, interleukin 5 and 8, and GRO/KC, which was associated with increased serum antioxidant capacity (Stoner et al. 2010). A number of clinical trails are in progress to test the effects of these berries on various human cancers (Stoner et al. 2007, 2008; Mallery et al. 2011).

Ginger Extract

One of the most widely used spices in Asian cooking, ginger (*Zingiber officinale Roscoe*) is an excellent source of several bioactive phenolics, such as gingerols, paradols, shogaols, and gingerones (Nigam et al. 2010; Oyagbemi et al. 2010; Shieh et al. 2010; Karna et al. 2012). Ginger has caught extensive attention due to its antioxidant, anti-inflammatory, and antitumor activities for both cancer therapy and prevention (Shukla and Singh 2007; Pereira et al. 2011; Baliga et al. 2012; Chen et al. 2012). Preclinical studies carried out in the last decade have shown that ginger and its phytochemicals dehydrozingerone and zingerone possess chemopreventive effects in experimental animals and in cultured cells in vitro (Kim et al. 2007; Baliga et al. 2012). For example, whole-ginger extract inhibited the growth and progression of prostate cancer PC-3 cell xenografts in nude mice by approximately 56% through reduction of cancer cell proliferation and induction of widespread apoptosis, but it had no detectable toxicity in normal and rapidly dividing gut tissues and bone marrow (Karna et al. 2012). Ginger containing [6]-gingerol induced apoptosis in a benzo[a]pyrene-induced mouse skin tumorigenesis model, which was associated with modulation of p53 expression and involvement of the mitochondrial signaling pathway (Nigam et al. 2010). Administration of 6-gingerol greatly enhanced the number of tumor-infiltrating lymphocytes in murine tumors (Ju et al. 2012). The ginger extract [6]-paradol had chemopreventive and antioxidant efficacy in DMBA-induced hamster buccal pouch carcinogenesis (Suresh et al. 2010). Ginger ingredients also inhibited development of a diethylnitrosoamine-induced premalignant phenotype in a rat hepatocarcinogenesis model (Mansour et al. 2010) and suppressed skin tumor initiation and promotion stages in ICR mice (Murakami et al. 2004). A tropical ginger sesquiterpene, zerumbone, inhibited colon and lung carcinogenesis in mice (Kim et al. 2009b). The extract 6-dehydrogingerdione induced cell cycle arrest and apoptosis by regulating reactive oxygen species/c-Jun N-terminal kinase pathways in breast cancer cells (Hsu et al. 2010). [6]-gingerol-induced reactive oxygen species regulated the mitochondrial cell death pathway in human epidermoid carcinoma cells (Nigam et al. 2009) and in human colorectal cancer cells (Lee et al. 2008). Ginger root extract can inhibit COX-2 activity in human colon mucosae or skin mucosa (Kim et al. 2005; Zick et al. 2011); MMP9 and NF- κ B/Snail activity in breast cancer cells (Ling et al. 2010; Hsu et al. 2012); and release of cytochrome *c* in tumor cells

(Rhode et al. 2007; Oyagbemi et al. 2010). Ginger extract also inhibited human telomerase reverse transcriptase and c-Myc expression in lung cancer cells (Tun-tiwechapikul et al. 2010). Ginger may bind to human serotonin receptors, which may influence gastrointestinal function (Riyazi et al. 2007; Nievergelt et al. 2010). To date, preclinical and animal studies have shown that ginger extract is a promising cancer preventive agent (Baliga et al. 2011), but further clinical studies are warranted to assess the efficacy and safety of ginger.

Broccoli Extract: Sulforaphane

Naturally occurring sulforaphane from broccoli or other cruciferous vegetables, such as Brussels sprouts, cabbage, and cauliflower, has been extensively studied for cancer prevention activities (Brennan et al. 2005; Eberhardt et al. 2005). In particular, sulforaphane enhanced the protection and repair of gastric mucosa against oxidative stress in vitro and demonstrated anti-inflammatory effects on *Helicobacter pylori*-infected gastric mucosae in mice and human subjects (2011), and consumption of broccoli sprouts effectively inhibited *H. pylori* growth and reduced colonization and attenuated gastritis in *H. pylori*-infected mice and humans (Galan et al. 2004; Yanaka et al. 2009; Yanaka 2011). The risk of breast cancer in premenopausal women and gastrointestinal and prostate cancers were inversely associated with consumption of broccoli (Kristal and Lampe 2002; Giovannucci et al. 2003; Hara et al. 2003; Ambrosone et al. 2004). Intake of cruciferous vegetables improved the survival rate of patients with bladder cancer in an epidemiological study (Tang et al. 2010) and in animal experiments (Munday et al. 2008). Sulforaphane inhibited breast cancer stem cells in mouse xenografts and downregulated the Wnt/ β -catenin self-renewal pathway in breast cancer cells (Li et al. 2010). Sulforaphane or other broccoli sprout extracts inhibited 4-aminobiphenyl-induced DNA damage in bladder cells and tissues (Ding et al. 2010) and mouse skin tumorigenesis during the promotion stage (Dinkova-Kostova et al. 2006; Gills et al. 2006). Sulforaphane inhibited extracellular, intracellular, and antibiotic-resistant strains of *H. pylori* and prevented benzo[*a*]pyrene-induced stomach tumors (Fahey et al. 2002). Molecularly, sulforaphane-containing broccoli sprout extracts induced phase II detoxification enzymes in humans (Dinkova-Kostova et al. 2007). Sulforaphane blocked MMP production in articular chondrocytes (Kim et al. 2009a) and in breast cancer cells (Rose et al. 2005). Broccoli sprout extract potently activated mitochondria-mediated apoptosis and arrested cancer cells in the S and M phases (Tang et al. 2006). Sulforaphane also inhibited histone deacetylase activity or cell-cycle-related genes (such as cyclin D1 and cdk4) in precancerous and cancerous prostate epithelial cells (Wang et al. 2004; Myzak et al. 2006a) and in Apc-minus mice (Myzak et al. 2006a). Sulforaphane can prevent intestinal polyposis in ApcMin/+ mice by regulating the expression of p-JNK, p-ERK1/2, and p-Akt (Hu et al. 2006). However, clinical trials are needed to test the true cancer prevention activities of sulforaphane and other broccoli extracts, especially in combination with other phytochemicals.

Phytochemicals Used in Clinical Trials

Although a great number of *in vitro* and animal studies have demonstrated the usefulness of various phytochemicals against tumors, a very few have successfully translated into clinical trials (Table 1 and reviews in Taylor and Greenwald 2005; Thomasset et al. 2007; Russo et al. 2010; Jin et al. 2012; Kumar et al. 2012). The reasons for failure include the lack of the phytochemical's bioavailability (i.e., achieving a sufficient concentration in targeting tissues), a clear or suitable molecular target, and multiple gene targets (see below). In addition, cancer prevention usually takes a very long time (years, not months) to show an effect on a cancer incidence rate. Thus, well-studied phytochemicals with clear molecular targets could help us to translate results into clinical trials. The following section discusses phytochemicals that are used in clinical chemoprevention trials.

β -carotene

The cancer preventive effects of β -carotene have been tested in a randomized clinical trial in patients with recent nonmelanoma skin cancer who received 50 mg of β -carotene or placebo to determine the occurrence of new nonmelanoma skin cancer (Greenberg et al. 1990). The trial did not observe any difference in nonmelanoma skin cancer occurrence between intervention and placebo group after five years of follow-up. The results did not show any efficacy either for patients whose initial plasma β -carotene level was in the lowest quartile or those who currently smoked. In another multicenter, randomized, double-blind, placebo-controlled trial, a combination of β -carotene and vitamin A was tested on the incidence of lung cancer and cardiovascular diseases (Omenn et al. 1996). This study included participants who were smokers, former smokers, and workers exposed to asbestos. The results indicated that the active-treatment group had 28% increased risk of lung cancer than the placebo group. They did not observe any statistically significant differences in the risks of other types of cancer. The relative risk of death from any cause including lung cancer and cardiovascular disease was higher in the active-treatment group. The investigators concluded that combination of β -carotene and vitamin A did not show any benefit and observed a potentially adverse effect on the incidence of lung cancer and on the risk of death from lung cancer and/or cardiovascular disease among smokers and workers exposed to asbestos (Omenn et al. 1996).

Another clinical trial tested β -carotene and α -tocopherol (vitamin E) to assess the preventive effect on lung and other cancers (Albanes et al. 1995). In this study, male cigarette smokers were randomly assigned to receive β -carotene (20 mg), α -tocopherol (50 mg), β -carotene and α -tocopherol, or placebo daily for five to eight years. The results suggested that β -carotene treatment did not reduce the cancer incidence at any of the major sites. In contrast, the investigators observed an increased incidence of lung, prostate, and stomach cancer. It is interesting to note, however, that investigators observed fewer incidence of the prostate, colon, and rectum cancers, but more cancers of the stomach, in the vitamin E intervention group (Albanes et al. 1995).

Table 1 Clinical Studies of Phytochemicals in Prevention or Control of Human Cancers. (See more in reviews of Taylor and Greenwald 2005; Thomasset et al. 2007; Russo et al. 2010)

Authors (years)	Phytochemicals or Extract	Type of Cancer	No. of Patients	Phase	Dose	Duration	Chemoprevention outcome
Greenberg et al. 1990	β -carotene	Skin cancer	1,968	III	50 mg	5 years	No difference vs. placebo
Omenn et al. 1996	β -carotene and vitamin A	Lung cancer	18,314	III	30 mg of β -carotene, 25,000 IU retinol	4 years	Increased risk of lung cancer in smokers vs. placebo
Albanes et al. 1995	β -carotene and vitamin E	Lung cancer	29,133	III	20 mg β -carotene, 50 mg vitamin E	5–8 years	Negative
Nguyen et al. 2012	EGCG	Prostate cancer	50	II	800 mg	3–6 weeks	Negative
Ahn et al. 2003	Green tea extracts	Cervical cancer	51	II	200 mg	Up to 12 weeks	Positive
Johnson et al. 2010	Green tea	Prostate cancer	50	II	200 mg	3–6 week	Positive
Bettuzzi et al. 2006	Green tea	Prostate cancer	60	II	600 mg/day	1 year	Positive
McLarty et al. 2009	Green tea	Prostate cancer	26	II	800 mg/day	2 weeks	Positive
Khan et al. 2012	Soy isoflavone	Breast cancer	98	IIB	One capsule/day	6 months	Biomarker trial, negative
Maskarimec et al. 2011	Soy isoflavone	Breast cancer	96	II	50 mg/day	6 months	Biomarker trial, negative
Miyana et al. 2012	Isoflavones	Prostate cancer	158	II	60 mg/day	1 year	Positive
Pendleton et al. 2008	Isoflavones	Prostate cancer	20	II	47 mg \times 3/day	1 year	Positive
Messing et al. 2012	Genistein	Bladder cancer, biomarker study	59		300 or 600 mg/day	2–3 weeks	Positive biomarker study
Dhillon et al. 2008	Curcumin	Pancreatic cancer	25	I	8,000 mg/day	8 weeks	Some activity in pancreatic cancer patients
Cruz-Correa et al. 2006	Curcumin	Familial adenomatous polyposis	5	I	480 mg curcumin	6 months	Positive
	quercetin				20 mg quercetin \times 3/day		
Carrall et al. 2011	Curcumin	Colon cancer	44	Ila	2 or 4 g per day	30 days	Positive biomarker study
Kuecik et al. 2001	Lycopene	Prostate cancer	26	II	15 mg \times 2/day	3 weeks	Positive

Green Tea and EGCG

A phase I pharmacokinetic study determined the systemic availability of green tea catechins after a single oral dose administration of EGCG and polyphenon E (Chow et al. 2001). Five healthy subjects were randomly assigned to receive 200, 400, 600, or 800 mg based on EGCG content. The study did not find significant difference in the pharmacokinetic characteristics of EGCG between the two study medications. Maximum plasma concentration of EGCG after the 800-mg dose was significantly higher than those after the 200- and 400-mg doses. The investigators concluded that the two-catechin formulations resulted in similar plasma EGCG levels. They also observed that conjugated forms of EGC and EC were present in the body after the polyphenon E administration. The investigators suggested that availability of a higher level of systemic EGCG was due to saturable presystemic elimination of orally administered green tea polyphenols (Chow et al. 2001). Another recent phase IB dose escalation trial in women with a history of stage I to III hormone receptor-negative breast cancer of an oral green tea extract, polyphenon E (Poly E) 400, 600, 800 mg twice daily or matching placebo for 6 months. They observed 27% of patients had dose-limiting toxicity with 600 mg Poly E (Crew et al. 2012).

Another clinical study evaluated the green tea polyphenol level in prostate tissue to measure its effects on systemic and tissue biomarkers. Study subjects received either polyphenon E (containing 800 mg of EGCG) or placebo daily for 3 to 6 weeks prior to prostate cancer surgery (Nguyen et al. 2012). The investigators observed green tea polyphenol levels in the prostatectomy tissue were low to undetectable. However, they found nonsignificant changes in serum prostate-specific antigen, serum insulinlike growth factor axis and oxidative DNA damage in blood leukocytes. They did not observe differences in cell proliferation, apoptosis, and angiogenesis markers in the prostatectomy tissue between treatment and placebo arm. The investigators concluded that low bioavailability or bioaccumulation of green tea polyphenols in prostate tissue moderately changes systemic and tissue biomarkers after 3 to 6 weeks of administration (Nguyen et al. 2012).

An additional study tested the efficacy of green tea extracts in a form of ointment or capsule in patients with human papilloma-virus–infected cervical lesions—chronic cervicitis, mild dysplasia, moderate dysplasia, or severe dysplasia. There was a statistically significant difference in response rate for treatment with green tea extracts compared to untreated controls. Results of this study demonstrated that green tea extracts in the form of ointment and capsule are effective for treating human papilloma-virus–infected cervical lesions (Ahn et al. 2003).

Curcumin

Animal studies have demonstrated that curcumin inhibit carcinogenesis of murine skin, stomach, intestine, and liver. However, there are a limited number of clinical studies that evaluated its effect in humans (Bar-Sela et al. 2010). Curcumin has been

evaluated in a phase I trial of 25 patients with recently resected urinary bladder cancer, arsenic-related Bowen's disease of the skin, uterine cervical intraepithelial neoplasm, oral leukoplakia, and intestinal metaplasia of the stomach (Cheng et al. 2001). The results of this study demonstrated that curcumin was not toxic to humans up to 8,000 mg/day when taken by mouth for 3 months. Curcumin has been tested in a phase II trial in patients with advanced pancreatic cancer and concluded that oral administration of curcumin is well tolerated despite its limited absorption (Dhillon et al. 2008). The investigators also noted that curcumin had biological activity in some patients with pancreatic cancer (Dhillon et al. 2008). Another study demonstrated that a combination treatment with curcumin and quercetin in patients with adenomas in familial adenomatous polyposis can reduce the number and size of polyps after six months of treatment (Cruz-Correa et al. 2006).

Lycopene

In recent years, lycopene has drawn a lot of attention for its antioxidant properties and potential role in cancer prevention. High intake of lycopene has been shown to be inversely associated with the incidence of certain types of human cancers, including digestive tract, prostate, and cervix (Franceschi et al. 1994; Giovannucci 1999; Giovannucci et al. 2002; Garcia-Closas et al. 2005; Seren et al. 2008). Tomato product consumption may prevent disease progression in benign prostate hyperplasia and prostate cancer (Kim et al. 2003; Schwarz et al. 2008). Lycopene supplementation has been tested in a phase II randomized clinical trial in patients scheduled to have a radical prostatectomy operation. The results of this study showed that lycopene supplementation stabilized serum prostate-specific antigen levels in men with prostate cancer. However, data from other published randomized clinical trials are inconclusive as to whether lycopene can prevent benign prostatic hyperplasia or prostate cancer (Kucuk et al. 2001; Cassileth 2010).

Isoflavones

Epidemiological studies have reported an inverse association between dietary soy consumption and the risk of prostate, breast, and endometrial cancers. Genistein is the most active and abundant isoflavone found in soybeans and soy products. Genistein has selective estrogen receptor modulator properties similar to those of tamoxifen and raloxifene (Atmaca et al. 2008). Soy isoflavones have been shown to benefit patients with prostate cancer and rising serum prostate-specific antigen (PSA) levels (Hussain et al. 2003). A significant decrease in the serum PSA level in biochemically relapsed prostate cancer patients has been reported based on results from a phase II trial (Pendleton et al. 2008). Another randomized double-blind placebo control clinical trial with isoflavone showed significant decreases in COX-2 expression level but increases in p21 mRNA in prostate cancer tissues after

two weeks of treatment with isoflavones before prostatectomy. Genistein was also tested in a phase II cancer chemoprevention biomarker trial in presurgical bladder cancer patients. The results showed that the treatment was well tolerated and toxicities were primarily mild to moderate gastrointestinal or metabolic and usually not attributed to the study drug. The investigators did not observe differences in biomarker modulation (COX-2, Ki-67, activated caspase-3, Akt, p-Akt, MAPK, and p-MAPK) by genistein (Messing et al. 2012).

Conclusion and Future Directions of Research

Although epidemiological and experimental studies have demonstrated that a healthy lifestyle with a diet rich in fruits and vegetables could reduce the cancer incidence rate among humans, chemoprevention clinical trials using combinations of phytochemicals to target distinct intracellular events have not provided impressive data. Because human carcinogenesis is a multistep process, more studies need to be conducted to understand the underlying molecular mechanisms, which will provide clearer targets that regulate cancer initiation, progression, and metastasis. The failure of clinical chemoprevention trials with phytochemicals has been due to a lack of bioavailability or specific molecular targets. Thus, it is extremely important to combine phytochemicals to prevent human cancers based on our understanding of molecular mechanisms of tumorigenesis in order to produce synthetic lethality and high-throughput screening of phytochemicals with other agents to target multiple gene pathways. Chemists should be recruited and involved to modify the structure of chemopreventive phytochemicals for bioavailability or improve their biological activity.

Specifically, combining different phytochemicals could achieve even better cancer prevention results by targeting more than one distinct intracellular event rather than a single biological response. Moreover, a combination could lower the dose of a single agent for long-term use and thus reduce unwanted side effects. A number of such studies have shown additive and synergistic effects of combined agents (Torrance et al. 2000; Kumar et al. 2001; Ye et al. 2012). Recently, investigators proposed the idea of “synthetic lethality” for cancer prevention, previously used for cancer therapy, which involves a combination of agents that may not be effective on their own, to induce tumor cell apoptosis (Zhang et al. 2010). This approach could dramatically increase the efficacy of phytochemicals to induce premalignant and malignant cells to apoptosis while keeping normal cells intact. However, although synthetic lethality would work on more than one gene pathway for synergistic effects on apoptosis, it could be applied to only to a limited cancer type and may not be generalizable to other cancers (Wu and Lippman 2011). In addition, high-throughput screening could reveal more effective phytochemical combinations for future cancer prevention studies (Twaddle et al. 2002). These approaches may help in achieving the maximal effects of phytochemicals, with minimal side effects, in preventing human cancers.

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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

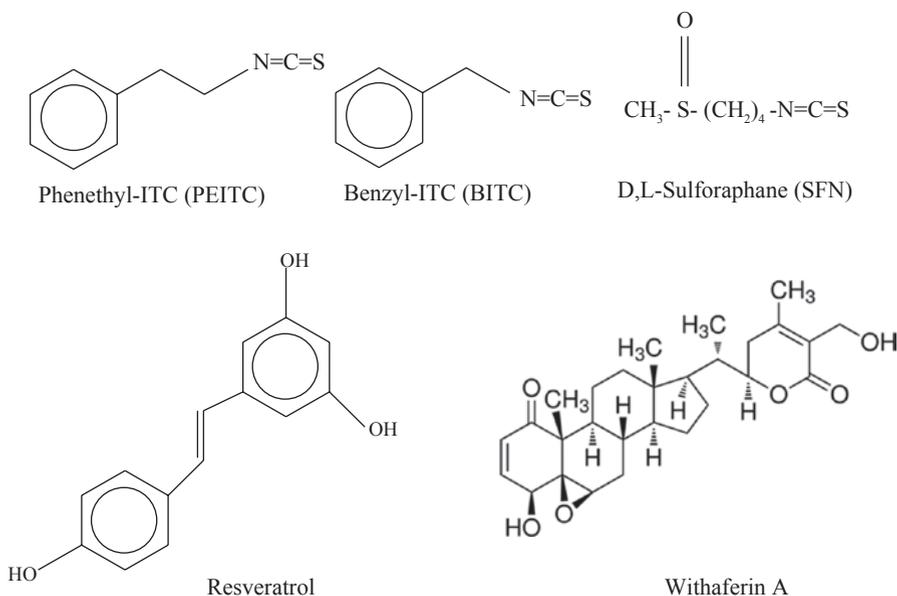


Fig. 1 Structures of the cancer chemopreventive phytochemicals reviewed in this chapter.

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; Bommarreddy 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 thiocarbamylation 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

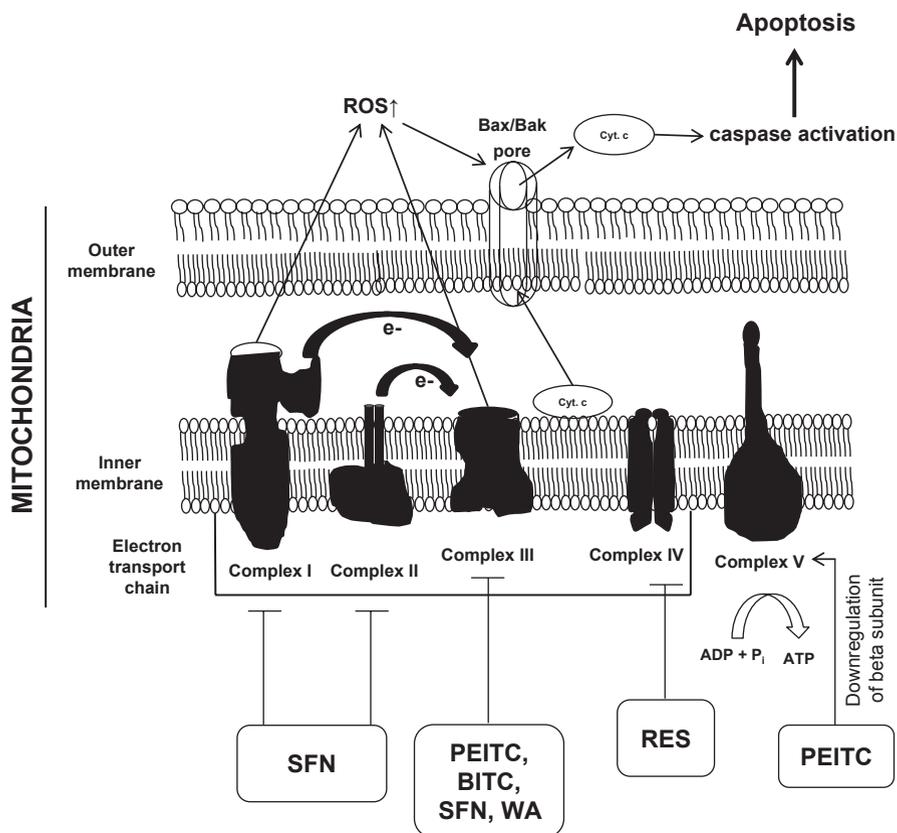


Fig. 2 A simplified cartoon depicting the role of mitochondria-derived reactive oxygen species in proapoptotic and chemopreventive response of selected phytochemicals. MRC targeted by these phytochemicals is shown.

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 NH₂-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 μ M 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 μ M) 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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Therapeutic Action of Phytochemicals on Cancer Stem Cells

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Abstract The cancer stem cell (CSC) concept has important implications not only for our understanding of carcinogenesis, but also for the development of cancer therapeutics. There is a growing body of preclinical evidence that cancer stem cells contribute to chemotherapy and radiation resistance in breast cancer. The use of drugs that interfere with stem cell self-renewal represents the strategy of choice, but also a great challenge because cancer stem cells and their normal counterparts share many pathways. Dietary compounds have been used in cancer prevention for decades, and some of these compounds target specific mechanisms that control CSC self-renewal. However, to date, no significant impact of CSCs on clinical outcome has been identified. The new paradigm imposed by the CSC model may change the way therapeutic effects are measured in clinical trials, stressing the effect on overall survival over just rapid tumor size reduction. In this chapter, we present the concept of cancer stem cell, mechanisms of conventional anticancer treatment resistance, and how dietary compounds may be used to target the self-renewal capability of CSCs.

Keywords Cancer stem cells • Self-renewal • Chemotherapy • Phytochemicals • Dietary compounds

Introduction

Cancer is the second most frequent cause of death in developed countries. The standard of care for systemic cancer treatment usually involves conventional chemotherapy where the choice of drugs is based upon tumor phenotype, patient condition, and whether the patient has previously responded to treatment, in the case where the tumor relapsed after a first line of treatment. Although most chemotherapeutic treatments induce tumor shrinkage, very often the tumor develops resistance and

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relapses. In recent years, it has become clear that most solid tumors show a hierarchical organization at the cellular level with a small population of cancer stem-like cells responsible for tumor initiation and maintenance, the so-called cancer stem cells (CSCs) or tumor initiating cells (TICs) for their ability to initiate tumors in immune-compromised animal models. The presence of cancer stem cells in tumors is likely one of the main reasons why current oncologic therapies are not very effective in preventing tumor progression, metastasis, and recurrence (Shafee et al. 2008; Tanei et al. 2009; Cirenajwis et al. 2010). Common chemotherapeutic drugs and radiotherapy often fail to eliminate these cells. Therefore, elimination of CSCs may become a necessary step for an effective cure, making CSCs as ultimate therapeutic target. Because CSCs are more resistant to conventional treatments than the bulk of differentiated tumor cells, the combination of CSC specific targeting agents with conventional chemotherapy will likely overcome tumor resistance and prevent tumor relapse, thus eventually will improve patient survival.

Medicinal plants have served as the source of therapeutic agents for many kinds of diseases including cancer. Natural compounds derived from fruits and vegetables (here onward referred as phytochemicals) have demonstrated their effectiveness in reducing the proliferation of tumor cells, both in vitro and in vivo. Epidemiological evidence has shown an association between certain dietary elements and a reduction of the incidence of cancer. In fact, some of the most common chemotherapeutic drugs are derived originally from plants, such as taxanes (paclitaxel, docetaxel, and others, derived from *Taxus brevifolia*), camptothecin (derived from *Camptotheca acuminata*), or vinca alkaloids (such as vincristine, derived from *Catharanthus roseus*). Some of the effects of phytochemicals may be directly related to the ability of the compounds to target cancer stem cell self-renewal. The aim of this chapter is to describe the current knowledge about the origin of cancer stem cells and how phytochemicals may target these rare cell populations, with special attention to breast cancer.

Cancer Stem Cells and Cancer Treatment

Tumors comprise heterogeneous populations of cells that have varying degrees of tumorigenic potential. Increasing evidence suggests that a biologically unique population of cancer stem cells exists in most neoplasms and may be responsible for tumor initiation, progression, metastasis, and relapse. Evidence that tumors arise from stem/progenitor cells has been obtained from leukemia (Bonnet and Dick 1997), breast (Al-Hajj et al. 2003), brain (Singh et al. 2003), colon (Ricci-Vitiani et al. 2007), and most other tumors (pancreas, melanoma, glioblastoma, ovary, liver, and prostate). However, the target cell for transformation that originates CSCs remains unknown. This could be a stem cell, a progenitor cell, or a terminally differentiated cell that acquires, through mutations and epigenetic changes, the stem cell self-renewing property. It is possible that any cell in the tissue cell hierarchy with proliferative capability could serve as a cancer-originating cell upon acquiring the changes that promote self-renewal and prevent postmitotic differentiation.

There are a number of reports using in vitro culture of tumor cells and animal models showing that CSCs are more resistant to conventional cancer therapies, thereby placing these cells at the root of tumor recurrence and metastases. Several preliminary reports have indeed shown that this is also the case for human cancer patients. In breast cancer, Li et al. (Li et al. 2008) showed that conventional chemotherapy increased the fraction of CD44^{high} CD24^{-/low} cells in a neoadjuvant setting of advanced breast cancer patients. Tanei et al. (Tanei et al. 2009) have shown that paclitaxel and epirubicin-based chemotherapy enriches for aldehyde dehydrogenase-1 positive cells in breast tumors; another marker for CSCs (Ginestier et al. 2007). CSCs from brain tumors expressing the neural stem cell surface marker CD133⁺ were resistant to standard chemotherapeutic drugs (Singh et al. 2004). Current therapeutic agents for the management of the cancer patient are directed towards rapidly proliferating cells, failing to address the mechanisms of self-renewal and tumor initiation, which are the mechanisms that define stem cell activity. Therefore, if CSCs have intrinsic different sensitivity to these agents, then treatment would not succeed in complete cancer eradication, and tumor shrinkage reflects the effect in rapid proliferating non-CSC cells. On the other hand, targeting just CSCs may not be sufficient as a cancer therapy because proliferating cells could also give rise to CSCs. Thus combined elimination of CSCs and non-CSCs may be the way to go for a complete cancer treatment. But, how are CSCs less sensitive to the conventional anticancer therapies? Several studies show that CSCs are relatively resistant to conventional antineoplastic agents, both in vitro and in vivo in animal models. For example, after treatment of TM40D breast cancer cells with paclitaxel/epirubicin, a common first-line treatment for breast cancer, most of the surviving cells expressed the CSC markers CD44⁺/CD24^{low}, also evidenced in tumor biopsies from treated breast cancer patients (Creighton et al. 2009). In colorectal cancer, human primary tumors transplanted into mice after treatment with oxaliplatin or irinotecan showed an increased fraction of cells with a CSC phenotype, compared with tumors before treatment or untreated tumors, and increased tumorigenicity (Dylla et al. 2008).

Mechanisms of Drug Resistance in CSCs

Stem Cells are not Actively Dividing Cells

Normal stem cell longevity is ensured by prolonged exit from the cell cycle, a mechanism that prevents the exhaustion of the replicative potential and limits DNA damage (Wilson et al. 2008). A similar mechanism is presumed to operate in CSCs, making these cells less sensitive to antiproliferative drugs, minimizing the exposure to DNA-damaging metabolic products. However, the existence of dormant CSCs has not been directly demonstrated and the cell cycle status of CSCs in homeostasis is still controversial.

Increased Expression of Antiapoptotic Proteins

Two major apoptosis-inducing pathways coexist in cancer cells. The extrinsic or receptor-mediated pathway is initiated upon engagement of one or several of the death receptor (DR) family, promoting the assembly of a multiprotein complex that ultimately activates the initiator caspase-8, that subsequently will activate the effector caspases (-3, and -7). The intrinsic pathway is initiated by the loss of outer mitochondrial membrane permeability and the release to the cytosol of proapoptotic mediators, mainly cytochrome *c* and Smac proteins. Cytochrome *c* binds to the scaffolding protein Apaf-1 that assembles a protein complex for the activation of the initiator caspase-9. Smac proteins are inhibitors of the inhibitors of apoptosis (IAPs) family, preventing their role as caspase activation blockers. The intrinsic pathway is engaged by a plethora of intracellular stimuli, mainly reflecting cell stress. Both pathways are tightly controlled by a complex network of proapoptotic and antiapoptotic proteins, such as the Bcl2 family proteins. CSCs have been reported to harbor multiple defects in the apoptosis-inducing machinery. For example, CD133-positive glioblastoma stem cells were reported to be resistant to Fas-induced apoptosis (extrinsic pathway), which was associated with the expression of a monomeric form of Fas protein (Bertrand et al. 2009). These cells also show higher expression of the inhibitor of apoptosis proteins XIAP and cIAPs compared to the CD133 negative population (Liu et al. 2006). CD133 positive cells in several tumor models showed increased expression of FLIP, an inhibitor of TRAIL (one of the DR) activation, making them more resistant to TRAIL-induced apoptosis (Zobalova et al. 2008). Increased expression of antiapoptotic Bcl2 family members have been described in glioma, breast, and colon cancer stem cells (Madjd et al. 2009; Kemper et al. 2012; Qiu et al. 2012), suggesting an alternative target to overcome treatment resistance in these tumors.

Increased Expression and Activity of Multifunctional Drug Efflux Channels from the ATP-Binding Cassette (ABC) Gene Family

Hematopoietic stem cells were described to express increased concentration of the transporters p-GP (MDR1, ABCB1) and BCRP (ABCG2; Lou and Dean 2007), and this feature has been exploited to isolate the stem cell population on the basis of dye exclusion (side population). However, stem cells from other tissues lack overexpression of these molecules. Besides, even if some transporters are overexpressed in stem cells, this may only explain resistance to the specific drugs that can be effluxed by them, not the wide resistance response observed including resistance to ionizing radiation.

Increased Expression of Detoxifying Machinery

The family of enzymes aldehyde dehydrogenase (ALDH) is involved in detoxification of intracellular aldehydes. In particular, ALDH1A1 and ALDH3A1 isozymes

have been shown to play important functional roles in normal stem cells able to metabolize chemotherapeutic agents, such as cyclophosphamide (Sladek et al. 2002). ALDH activity, detected by the conversion of the metabolic substrate Aldefluor®, is commonly used as a marker for CSCs (Charafe-Jauffret et al. 2009) and high ALDH1 expression has been shown to correlate with poor prognosis in breast cancer patients (Ginestier et al. 2007).

Lack of Hormone Receptors

CSCs in hormone-dependent cancers, such as breast cancer or prostate cancer, have been shown not to express hormone receptors [estrogen receptor (ER) and progesterone receptor (PR) for breast cancer; and androgen receptor (AR) for prostate cancer]. Therefore, CSCs are not being affected by drugs targeting hormone receptors (such as tamoxifen).

Targeting CSCs seems the right approach to cure the patient effectively, assuming that the CSC population is stable over time and that the CSC phenotype is intrinsic cell autonomous features not attainable by differentiated non-CSC cells. Results from our group and others, however, suggest that this may not be the case and point towards a more flexible and dynamic CSC population (Mani et al. 2008; Iliopoulos et al. 2011; Leis et al. 2012). If this is the case, then therapy must be directed not towards CSCs, but towards the molecular mechanisms responsible for the activation of CSCs at tumor initiation and during tumor progression. In particular, stemness-associated pathways, such as those involved in the induction and maintenance of pluripotency, are promising targets for anti-CSCs drug development.

Functional Assessment of Cancer Stem Cell Activity

Application of stem cell biology to cancer research has been limited by the lack of simple methods for identification and isolation of normal and malignant stem cells. Assays commonly used to assess stem cell activity in tumors are described below.

Cell Surface Markers

In 1997 Dick et. al. demonstrated that human leukemias are driven by a small population of cells with the CD34⁺ and CD38⁻ phenotype. Transplantation to humanized NOD/SCID mice at a number as few as 100 cells are capable of regenerating the original tumor (Bonnet and Dick 1997). Clarke and Wicha extrapolated this concept to solid tumors demonstrating that human breast tumors have a population of cells with stem cell properties. Using flow cytometry based on cell surface markers they differentiated the tumorigenic (tumor initiating) from the nontumorigenic cancer cells, identifying the tumorigenic cells as CD44⁺ CD24^{-/low} Lin⁻. This population has

capacity to generate the phenotypic heterogeneity found in the initial tumor when transplanted to humanized NOD/SCID mice (Al-Hajj et al. 2003). Since then, specific surface markers expressed on CSCs but not on the bulk of the tumor have been identified on a variety of cancers, including brain cancers (CD133⁺), prostate cancer, melanoma, multiple myeloma, colon (CD133⁺), pancreatic, and head and neck cancers. Nevertheless, there is still not a single CSC specific marker, likely due to functional plasticity of this population. Thus, a cell that shows CSCs activity may not express a CSC-designated marker although functionally capable of initiating tumors. This complicates pathological evaluation of CSC content from natural tumor samples.

Side Population (SP)

ATP binding cassette (ABC) transporters represent a family of proteins with the capacity to bind ATP as an energy source to transport endogenous or exogenous molecules across the cellular membrane). Some of these proteins, such as the proteins encoded by MDR, MRP, and BCRP1, contribute to drug resistance and subsequent recurrence in cancers (Hadnagy et al. 2006). BCRP1 excludes the fluorescent dye Hoechst 33342 that universally binds to the AT-rich regions of the minor groove of DNA, identifying a side population (SP) of cells, which is enriched for cells with stem cell characteristics. A variety of established cancer cell lines, which have been maintained in culture for decades, and also tumors contain a small SP. These SP cells, but not non-SP cells, self-renew in culture, are resistant to anticancer drugs, have the capacity to form tumors when transplanted in vivo and can be identified as the “side” of the bulk of the Hoechst 33342 positively stained cells in fluorescence-activated cell sorting (FACS) analysis plots. However, this staining is technically challenging and not always reproducible; in addition, DNA intercalating agents affect the viability of the cells in subsequent cultures, limiting the application of this procedure. On the other hand, this is a functional parameter not limited to the expression of a particular marker on the cell surface.

Aldehyde Dehydrogenase 1

Aldehyde dehydrogenase 1 is an intracellular enzyme whose functions include the oxidation of toxic aldehyde metabolites to carboxylic acids like those formed during alcohol metabolism. It has been shown that ALDH1 activity enriches for cells with stemlike properties in a variety of solid malignancies (Ginestier et al. 2007). This enhanced detoxifying activity, besides its use as a marker for stem cells, may relate to the lower sensitivity of stem cells to certain chemotherapeutics, such as cyclophosphamide (Sladek et al. 2002). Interestingly, ALDH activity does identify a different population from, for example, CD44⁺ CD24^{-/low} in breast cancer, pointing to the existence of different CSC populations or several functional states on CSCs. As ALDH1 activity has been used as a common marker for both normal and malignant stem and progenitor cells, commercial kits have been released to identify and isolate cells with high ALDH1 activity.

Sphere Formation Assay

Derived from the neural stem cell field, CSCs when cultured in serum-free restricted medium with proper growth factors preventing attachment to a substrate, can form floating spheroidal aggregates (tumorospheres) that are enriched in CSC (Dontu and Wicha 2005). Hepatoma cell lines, squamous cell carcinoma cell lines, or head and neck squamous carcinoma cell lines, among others, form nonadherent tumor spheres in culture that possess CSC properties. In breast carcinoma cell line MCF7, the mammosphere assay has been demonstrated to enrich and propagate cells with enhanced tumor initiating ability (Deleyrolle et al. 2011). This assay is used as a stem-cell-like functional assay that allows the propagation of mammary epithelial and breast tumor cells in an undifferentiated state based on their ability to proliferate in suspension and as a functional *in vitro* assay for cancer stem-like specific drug screening. A limitation of the sphere assay relates to whether this assay properly identifies the frequency of *in vivo* quiescent stem cells as opposed to measuring cells that adapt or can act as a proliferating mammary stem cell *in vitro*. Furthermore, not every cell line, despite its tumor-initiating ability, can form tumor spheres in culture, raising questions about the restrictions imposed on cell growth in this assay.

Mice Xenografts

Currently, the gold standard functional assay to demonstrate tumor-initiating ability consists of hetero-transplantation of human cancer cells into immunodeficient mice. This xenograft model has been used to study cancer pathogenesis and drug development for several decades (Morton and Houghton 2007), and with the development of FACS analysis, self-renewal capacity of a subpopulation with a given cell surface phenotype is commonly assessed using limiting dilution cell transplantation into immune-deficient mice and then scored for tumor engraftment. Mice xenograft models can also be utilized to recapitulate a primary tumor from biopsy samples. Primary tumors are minced and enzymatically digested. Then primary tumor-derived cells are transplanted into mice, either under the skin or into the organ type in which the tumor originated, at varying cell densities. The developing time of the tumor will depend on the number of cells inoculated. This assay is costly and very low throughput, limiting its use to laboratories with dedicated animal facilities.

Zebrafish

The mice xenograft model presents several caveats at a practical level, such as expensive animal facilities, number of animals used in each experiment, and the length of time to tumor formation. Zebrafish have been widely used in preclinical tests and drug screening, as well as toxicity assays for a variety of reasons: fish are inexpensive to maintain, breed in large numbers (100–300 embryos per week/couple),

develop rapidly *ex vivo*, embryos are transparent, have short generational cycles (2–3 months), are immunodeficient until day 11 postfertilization, require a small amount of drugs per experiment, small in size, optically clear during development, and amenable to genetic manipulation. Recently, tumor cell xenografts into 2 days-postfertilization zebrafish embryos have proved useful to assess stem cell features (Eguiara et al. 2011). Therefore, zebrafish xenografts may represent a better alternative to medium throughput drug screening *in vivo*, not achievable using mice.

Molecular Targets of Phytochemicals in Cancer Stem Cells

The molecular mechanisms that control self-renewal of cancer stem cells are essential elements for tumor survival and propagation. Multiple signaling pathways (Fig. 1) have been identified including the Wnt/ β -catenin, Hedgehog (Hh), and Notch and PI3K-Akt signaling pathways (Beachy et al. 2004). Although genes involved in these pathways are expressed in normal stem cells, they are frequently mutated or aberrantly activated in almost all cancers. As mentioned in Fig. 1, dietary phytochemicals are natural products target multiple signaling pathways in CSCs, such as Wnt signaling in breast cancer (Kakarala et al. 2010) or side population in brain tumors (Fong et al. 2010). It would be interesting to determine if these compounds have differential effects on CSCs, and if so, understanding the mechanism of action of phytochemicals would lead to the development of novel therapeutic drugs for cancer treatment. Some of the phytochemicals possessing anti-CSCs activities are mentioned below.

Curcumin

Curcumin is a well-known dietary polyphenol present in an Indian spice called *Curcuma Longa* usually used in the preparation of curry. It has anticancer activity both *in vitro* and *in vivo* models (Epstein et al. 2010). Unfortunately, it also affects cell proliferation through cell cycle arrest and cytotoxicity in both normal and transformed cells (Karmakar et al. 2006). It has been described that curcumin affects many signaling pathways (Fig. 1) related to apoptosis, proliferation, stem cell self-renewal, and epithelial-to-mesenchymal transition (EMT), as well as Wnt/ β -Catenin and Notch pathways (Yan et al. 2005; Karmakar et al. 2006; Ryu et al. 2008; Kakarala et al. 2010; Yang et al. 2012).

Piperine

Piperine is a dietary polyphenol, isolated from black and long peppers, which has been reported to reduce cancer incidence in animal models (Pradeep and Kuttan

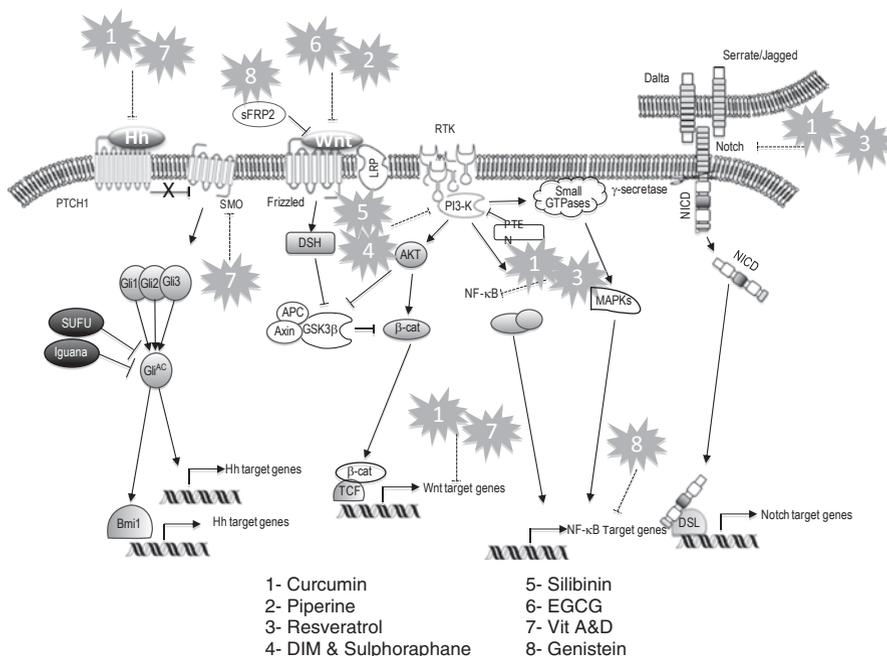


Fig. 1 Schematic representation of signaling pathways that operate in CSCs and where the described phytochemicals exert their effect

2002; Selvendiran et al. 2004). It was reported that piperine altered cancer stem cell self-renewal by inhibiting the ability of stem cells to grow as floating mammospheres and reducing the cell population that shows increased ALHD activity (Kakarala et al. 2010), without affecting the differentiated cells in the culture. The specific mechanisms operating in cancer stem cell self-renewal targeted by piperine are not currently known.

Resveratrol

Resveratrol, another polyphenol is an ingredient of red wine, stops breast cancer cell growth by blocking growth stimulating effect of estrogen (De Amicis et al. 2011). This paper suggests that resveratrol is able to counteract the malignant progression by inhibiting the proliferation of hormone-resistant breast cancer cells. This has important implications for the treatment of women with breast cancer resistant to hormonal therapy. It has also been described as a DNA demethylating agent in breast tumors and breast carcinoma cell lines (Zhu et al. 2012). It has recently been described that resveratrol synergizes with curcumin to inhibit colon cancer growth in mouse models, suggesting a better response to chemopreventive agents (Majumdar et al. 2009).

Cruciferous Vegetable Derived Compounds

Cruciferous vegetables such as broccoli, cabbage, kale, Brussels sprouts, and radish, have been shown to contain absorbable 3,3'-diindolylmethane (DIM), which prevent cancer (Bradlow et al. 1999). Sulphoraphane is another bioactive compound that is abundant in cruciferous vegetables and was shown to block mammosphere formation in breast carcinoma cell lines in vitro and decrease tumor size in mouse xenograft models, associated with a reduction of the stem cell marker ALDH (Li et al. 2010), although it is not clear what pathways are targeted.

Silibinin

Silymarin and its major constituent silibinin, are extracted from the medicinal plant *Silybum marianum* (milk thistle) and has traditionally been used for the treatment of liver diseases. Recently, these orally active flavonoid agents have also been shown to exert significant antineoplastic effects in a variety of in vitro and in vivo cancer models, including skin, breast, lung, colon, bladder, prostate, and kidney carcinomas (Hogan et al. 2007) due to induction of apoptotic death. More studies are required in order to determine whether it has any effects on CSCs.

Epigallocatechin-3-Gallate (EGCG)

(-)-Epigallocatechin-3-gallate (EGCG) is a bioactive polyphenolic compound present in green tea, which is one of the most widely consumed beverages in the world. Epidemiological studies suggest an association between green tea consumption and cancer prevention agents (Landis-Piwowar et al. 2007). It has been extensively described as a Wnt pathway regulator, one of the key pathways controlling stem cell self-renewal in breast cancer and colon cancer (Bose et al. 2007). EGCG induces HMG box-containing protein 1 (HBPI) transcriptional factor, which is a recognized suppressor of Wnt signaling (Kim et al. 2006). Another described effect of EGCG is altering chromosomal structure through reduction in Bmi-1 levels (Balasubramanian et al. 2010). Bmi-1 is highly expressed in cancer stem cells such as leukemia, neuroblastomas, and skin cancer, accompanied by the decreased expression of p16^{Ink4a} and p19^{Arf} tumor suppressor genes. Taken together, these studies support the further evaluation of EGCG in CSCs.

Vitamin A and D

One of the isoforms of vitamin D, cholecalciferol (Vitamin D3), was demonstrated to block Hedgehog-dependent signaling in breast cancer cell lines through binding

to Smo, although it did not show effects on tumor growth in vivo (Bijlsma et al. 2006; Bruggemann et al. 2010). Vitamin D can also interfere with the oncogenic mechanisms of β -catenin activity (the effector in the canonical Wnt signaling pathway) through a dual mechanism: vitamin D can modulate the expression of the Wnt signaling inhibitors DKK1 and DKK4 in colon cancer cells (Aguilera et al. 2007; Pendas-Franco et al. 2008), and on the other hand promote the translocation of β -catenin from nucleus to plasma membrane and thereby inhibit the expression of β -catenin-responsive genes through association with the Vitamin D receptor (VDR) (Palmer et al. 2001).

Genistein

Genistein is an isoflavone which is the major bioactive compound extracted from soy. Epidemiological evidence suggests that soy consumption decreases the risk of cancer (Messina et al. 1994). As other isoflavones, genistein has been explored as an angiogenesis inhibitor. Besides, various studies have found that moderate doses of genistein have growth inhibitory effects on prostate, brain, breast, and colon cancer (de Lemos 2001; Morito et al. 2001; Hwang et al. 2009; Nakamura et al. 2009; Das et al. 2010; Sakamoto et al. 2010). Regarding cancer stem cells, lifetime feeding of genistein (250 mg/kg per day) to rats increased expression of the Wnt signaling antagonist secreted frizzled-related protein 2 (sFRP2) and thus might account for a reduction in stem cell self-renewal (Su et al. 2007). It is interesting that downregulation of sFRP2 is a frequent event in breast cancer (Suzuki et al. 2008).

Clinical Trials Related to CSCs and Future Perspectives

A great proportion (70%) of drugs tested in oncology fail in randomized phase III clinical trials, despite extensive evidence in animal models showing therapeutic effect. The efficacy of antitumor agents in phase II clinical trials is commonly evaluated following RECIST (Response Evaluation Criteria In Solid Tumors) rules that define when cancer patients improve (“respond”), stay the same (“stable”), or worsen (“progression”) during treatments. Since the bulk of tumor cells (non-CSCs) constitute most of the tumor mass, efficacy mainly reflects the ability to kill those non-CSCs. Thus it is not tumor size reduction, but instead complete response (CR), that is a valid endpoint when associated with reduced recurrence rate. An agent that only targets CSCs is predicted to show only moderate effect on tumor size (therefore scored as a failure) but would have dramatic effect preventing tumor recurrence. On the other hand, an agent that targets the bulk of tumor cells but not CSC self-renewal will initially show good clinical response but will not prevent recurrence. Such a trial may result in a failure because of evidence

of tumor progression. Therefore, innovative clinical trial designs are required to assess efficacy of these drugs with appropriate biological and clinical endpoints. For example, 80% of breast cancer patients show good clinical outcome in five years, therefore, a clinical trial designed against breast cancer stem cells would be directed to patients that have failed a second or third line of treatment (usually chemotherapy) where they are less likely to respond to any treatment. It will be lengthy process and involve a significant number of patients, thus the cost would be huge. It is necessary to introduce recurrence in the adjuvant setting to identify effective CSC targeting agents. For new agents that are tested against CSCs, in order to expedite their approval by the regulatory authorities, it might be desirable to seek niche indications where rapid clinical endpoints can be assessed. For example, small-cell-lung-carcinoma typically responds well to first-line chemotherapy, however, most patients relapse within 12 months. Therefore, a valid indication would be to treat with anti-CSCs agents just after the first line of chemotherapy, where the endpoint would be to look for relapse-free survival. Once a novel agent is approved, its transition to other indications is faster. Another possibility would be to combine current chemotherapeutic treatment with anti-CSC phytochemicals, however, this scenario would complicate the design of clinical trials. Only if such a phytochemical is proved to lower the resistance threshold of a known chemotherapeutic would it be advisable to use them in combination.

Dietary phytochemicals are considered attractive alternatives for development in cancer chemoprevention. As outlined before, resveratrol, piperine, genistein, or curcumin have undergone extensive mechanistic and preclinical efficacy investigation, although their clinical use is still very scarce. As opposed to chemical anticancer drugs, that are designed to act on specific targets, dietary agents exert a plethora of actions with an unknown hierarchy of biological importance, lacking a clear correlation between effect and mechanistic information. Moreover, as chemopreventive agents, clinical trials involve lengthy periods of time to assess efficacy, as well as a significant number of patients. Dose determination is also tricky, as effective doses used *in vitro* usually are several orders of magnitude above the typical dose of the phytochemical found in the ordinary diet, with potential for appearance of toxic effects *in vivo*. Of course, any toxic effect for chemopreventive agents is unacceptable. Altogether, these caveats make clinical trials with dietary compounds unattractive to trial sponsors, which explains the lack of funding.

Nevertheless, it would be very promising to study dietary compounds' efficacy against CSCs. Given that these diet-based compounds are usually multitargeted, they may mediate other cellular events, for example, induction of CSC differentiation and sensitization of CSCs to chemotherapeutic agents, in addition to their potential impact on self-renewal signaling. No specific clinical trial has been designed thus far to assess phytochemicals effect on CSCs, although numerous trials are actively seeking to investigate their use as more effective strategies for cancer treatment, and to reduce cancer resistance and recurrence, thus improving patient survival.

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Phytochemicals, microRNAs, and Cancer: Implications for Cancer Prevention and Therapy

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Abstract Phytochemicals are bioactive plant compounds that have drawn significant attention as preventive and therapeutic agents against cancer. With emerging evidence, it is now becoming clear that phytochemicals confer anticancer activities by regulating the expression of microRNAs (miRNAs/miRs), which are functionally involved in cancer pathobiology. miRNAs represent a novel class of gene regulators, whose altered expression by phytochemicals leads to modulation of pathologically relevant target genes, and thus affects tumor cell growth and malignant properties. miRNAs also associate with and localize to mitochondria, suggesting that mitochondrial resident miRNAs may play a role in regulation of cell death in response to phytochemicals. In this chapter we provide a brief overview of various phytochemicals and cancer-associated miRNAs and discuss some of the available literature on miRNA regulation by phytochemicals as a mechanism(s) for their anticancer activities. We further discuss the role of miRNAs in cancer pathogenesis and their targeting by plant-derived natural compounds.

Keywords MicroRNAs • Phytochemicals • Cancer prevention and therapy • Phenolics • Carotenoids • Alkaloids • Organosulfurs

Introduction

Phytochemicals are naturally occurring compounds in plants; however, the term is generally used to refer to the plant-derived chemicals that are biologically active. Phytochemicals are present in numerous plant-based foods such as fruits, vegetables, grains, and beverages and have various applications. Several epidemiological and laboratory studies suggest that phytochemicals may reduce the risk of cancer because of their antioxidant and anti-inflammatory properties (Reddy et al. 2003; Block 1992; Steinmetz and Potter 1996; Cohen et al. 1999; Nichenametla et al. 2006).

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Thus far, more than 10,000 phytochemicals have been identified (Russo et al. 2010) and an interesting fact is that nearly 47% of FDA-approved anticancer drugs are derived from plants (Newman and Cragg 2007). Phytochemicals exert their anticancer effects through modulation of cell signaling pathways by directly or indirectly targeting cancer-relevant molecular targets (Russo et al. 2010).

MicroRNAs (miRNAs) are small regulatory noncoding RNA molecules (19–25 base pair size) that control gene expression through posttranscriptional mechanisms (Krol et al. 2010). miRNAs bind to the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs) and either cause their degradation or inhibit translation (Bhardwaj et al. 2010). It has been established that a single miRNA can target multiple mRNAs, whereas a single mRNA can be targeted by several different miRNAs (Srivastava et al. 2013; Pillai 2005). It is estimated that miRNAs regulate more than one-third of all human genes, which indicates that miRNAs can remarkably influence human biology. To date, more than 500 miRNAs have been identified in humans (Bhardwaj et al. 2010). miRNAs regulate various biological processes such as development, cell differentiation, migration, and survival, and their dysregulation causes various developmental defects (Srivastava et al. 2013; Sassen et al. 2008). Several studies have shown that miRNAs exhibit differential expression patterns in various human diseases, including cancer, and are functionally involved in disease processes (Calin and Croce 2006). Growing data suggest that phytochemicals modulate the expression of several cancer-associated miRNAs and thus have an impact on the pathogenesis of cancer (Ross and Davis 2011). This chapter summarizes some of the significant findings on the role of phytochemicals in modulating miRNAs and the implication of such findings in cancer prevention and therapy.

Role of MicroRNAs in Cancer

miRNAs critically influence the development and progression of cancer. They either act as growth promoters (oncogenic miRNAs; oncomiRs) or suppressors (tumor suppressor miRNAs; anti-oncomiRs). In addition, they can also affect tumor metastasis and associated cellular processes as well as the response of tumor cells to various therapies.

Oncogenic MicroRNAs (OncomiRs)

Oncogenic miRNAs are those miRNAs that promote tumor development by repressing the expression of tumor suppressor genes and/or by regulating the genes responsible for pathobiology of cancer (Zhang and Coukos 2006). These oncogenic miRNAs are overexpressed in a wide variety of cancers as a result of gene amplification and/or epigenetic and transcriptional dysregulation (Ha 2011). Expression profiling studies have shown that miR-155, a potentially oncogenic miRNA, is overexpressed in various

malignancies (Iorio et al. 2005; Greither et al. 2010; Lawrie 2007). Furthermore, overexpression of miR-155 has been shown to promote the growth of MDA-MB-231 breast cancer cells (Jiang et al. 2010) and is associated with low survival in pancreatic cancer patients (Greither et al. 2010). In other reports, the oncogenic role of the miR-17-92 cluster has also been demonstrated in various cancers (Diosdado et al. 2009; Hayashita et al. 2005). Ectopic expression of the miR-17-92 cluster enhances c-myc mediated tumor development in a mouse B-cell lymphoma model (He et al. 2005). Furthermore, upregulation of the miR-17-92 cluster enhanced tumorigenesis and angiogenesis (Dews et al. 2006). In two different reports, miR-21 was shown to be overexpressed in breast cancer and glioblastoma (Iorio et al. 2005; Papagiannakopoulos et al. 2008; Si et al. 2007). miR-21 acts as an oncogenic miRNA by suppressing apoptosis through activation of caspases in human glioblastoma cells (Chan et al. 2005). Furthermore, silencing of miR-21 is shown to diminish growth and induce apoptosis by downregulating Bcl-2 in breast cancer cells (Si et al. 2007). Thus, these studies represent some of the examples from a long list of oncogenic miRNAs that have been experimentally validated.

Tumor Suppressor MicroRNAs

miRNAs that inhibit tumor growth are known as tumor suppressor miRNAs. Emerging evidence suggests that tumor suppressor miRNAs are frequently downregulated in various cancers such as chronic lymphocytic leukemia, hepatocellular carcinoma, and breast, lung, and pancreatic cancer (Iorio et al. 2005; Yu et al. 2007; Calin et al. 2002; Li et al. 2008; Srivastava et al. 2011). Indeed, the first evidence of a functional role of miRNAs came from the identification of tumor suppressor miRNAs, miR-15 and miR-16 in chronic lymphocytic leukemia (CLL; Calin et al. 2002). Levels of miR-15a and miR-16-1 are inversely correlated with Bcl-2 expression and its overexpression induces apoptosis (Cimmino et al. 2005). In prostate cancer cells, restoration of miR-16 significantly reduced the cell growth (Takeshita et al. 2010). The let-7 family is one of the most studied tumor suppressor miRNAs, which is significantly downregulated in cancers of lung, breast, colon, and pancreas among several others (Yu et al. 2007; Zhang et al. 2007; Johnson et al. 2005). In a clinical study on lung cancer, decreased let-7 expression correlated with shorter postoperative survival (Takamizawa et al. 2004). Furthermore, let-7 has also been shown to regulate the RAS oncogene negatively (Johnson et al. 2005). In one of our recent studies, we have identified miR-150 as a potential tumor suppressor miRNA in pancreatic cancer (Srivastava et al. 2011). We have provided evidence that miR-150 is downregulated in pancreatic cancer and represses the expression of MUC4 oncoprotein by direct targeting. miR-150-mediated MUC4 downregulation in part resulted in suppression of growth and malignant potential in pancreatic cancer cells (Srivastava et al. 2011). In a study on mixed lineage leukemia (MLL), miR-495 was shown to be significantly downregulated and its overexpression inhibited cell viability under *in vitro* conditions and reduced leukemogenesis *in vivo* (Jiang et al. 2012). miR-34 is downregulated in many cancers and restoration of

miR-34 results in the cell-cycle arrest, inhibition of angiogenesis and malignant properties, and apoptosis (Chang et al. 2007; Javeri et al. 2013). Furthermore, the inhibitory effect of miR-34 on tumor-initiating cells has been also reported in pancreatic cancer (Ji et al. 2009).

MicroRNAs as Metastasis Modulators

Metastasis is the ability of the tumor cells to migrate and invade from one organ to another nonadjacent organ, and is a major cause of mortality in cancer patients. Thus far, several miRNAs have been identified to be involved in the process of metastasis. Ma and coworkers demonstrated, for the first time, the role of miRNAs in metastasis (Ma et al. 2007). Their study revealed upregulation of miR-10b in metastatic breast cancer cells, where it promoted cell migration, invasion, and metastasis under *in vitro* and *in vivo* conditions. Furthermore, the level of miR-10b in primary breast carcinoma was correlated with disease progression (Ma et al. 2007). In a separate study on breast cancer, miR-373 and miR-520c were shown to promote cancer cell migration and invasion by targeting CD44 expression (Huang et al. 2008). miR-373 or miR-520c overexpressing MCF-7 cells were able to develop metastatic nodules in mice and high levels of miR-373 were observed in clinical breast cancer metastasis specimens (Huang et al. 2008). In another study, restoration of miR-373 and miR-520c enhanced migration and invasion of prostate cancer cells (Yang et al. 2009). miR-335, miR-126, and miR-206 were also shown to act as metastasis suppressor miRNAs in breast cancer (Tavazoie et al. 2008). Re-expression of these miRNAs in breast cancer cells suppressed lung and bone metastasis (Tavazoie et al. 2008). Restoration of miR-126* in prostate cancer cell resulted in decreased cell migration and invasion (Musiyenko et al. 2008). The role of miR-183 and miR-126 in the inhibition of metastasis has been demonstrated in lung cancer (Wang et al. 2008; Crawford et al. 2008). Thus, all these findings indicate the role of miRNAs in the regulation of metastasis.

MicroRNAs in Therapy Resistance

In recent years, significant developments have been made for the treatment of cancer. However, cancer cells acquire chemoresistance, which is a major obstacle for the successful outcome of drug therapy. Growing studies indicate the role of miRNAs in anticancer drug resistance. In a tamoxifen-resistant breast cancer cell line, 8 miRNAs were found to be upregulated, and 7 were downregulated as compared to parental tamoxifen-sensitive MCF-7 cells (Miller et al. 2008). This study further suggested that overexpression of miR-221 and miR-222 in breast cancer cells contributed to tamoxifen resistance (Miller et al. 2008). In a separate study, it was shown that miR-451 regulated the expression of a multidrug resistance gene (MDR; Kovalchuk et al. 2008; Xia et al. 2008). In multidrug-resistant human gastric cancer cell line SGC7901/VCR, miR-16 and miR-15 were downregulated and their forced

expression sensitized the cancer cells to chemotherapeutic drug-induced apoptosis (Xia et al. 2008). Overexpression of miR-214 was shown to promote survival of ovarian cancer cells by imparting resistance to cisplatin (Yang et al. 2008). In other studies, let-7 and miR-200 were shown to enhance the radio- and chemosensitivity in lung and pancreatic cancer cells, respectively (Li et al. 2009; Weidhaas et al. 2007). The expression level of miR-221 and miR-222 was found to be elevated in TRAIL (tumor necrotic factor-related apoptosis-inducing ligand) -resistant non-small-cell lung cancer (NSCLC) cells, and it was demonstrated that these miRNAs were essential for maintaining the TRAIL-resistant phenotype (Garofalo et al. 2008).

Phytochemicals in Cancer Prevention and Therapy

Various phytochemicals have been used in different types of cancer for prevention and therapy. These phytochemicals impart their effect by interfering with stages of the carcinogenesis and drug resistance. To account for the anticancer effect, phytochemicals target various signaling nodes or molecular targets involved in cancer pathogenesis. In addition to their anticancer properties, phytochemicals have a high potential to be commercialized due to some basic advantages over synthetic drugs viz. low toxicity, ready availability, and cost-effectiveness (Deorukhkar et al. 2007; Lee et al. 2011). Therefore, there is a global demand that phytochemicals be developed as chemopreventive and therapeutic agents (Harvey 2008; Singh 2007).

Major Types of Bioactive Phytochemicals

Phytochemicals are categorized into different classes based on their chemical structure (Fig. 1). Below we describe these classes, their distribution, and potential biological activities.

Phenolics

Phytochemicals containing one or more aromatic rings with hydroxyl group(s) are termed phenolics (Wahle et al. 2010). Phenolics are secondary metabolites of plants and play essential function in development, reproduction, and defense, as well as provide color to plants (Dai and Mumper 2010). In addition to their roles in plants, phenolic compounds also confer several health benefits to humans associated with various illness (Dai and Mumper 2010). Among the fruits consumed in the United States, cranberry possesses the highest phenolic content followed by apple, red grape, strawberry, pineapple, pear, banana, orange, peach, and lemon (Sun et al. 2002). Among various vegetables consumed in the United States, broccoli has the highest total phenolic content followed by spinach, yellow onion, red pepper, cabbage, carrot, cucumber, potato, and lettuce (Chu et al. 2002). Phenolic phytochemicals are further subdivided into flavonoids, phenolic acids, coumarins,

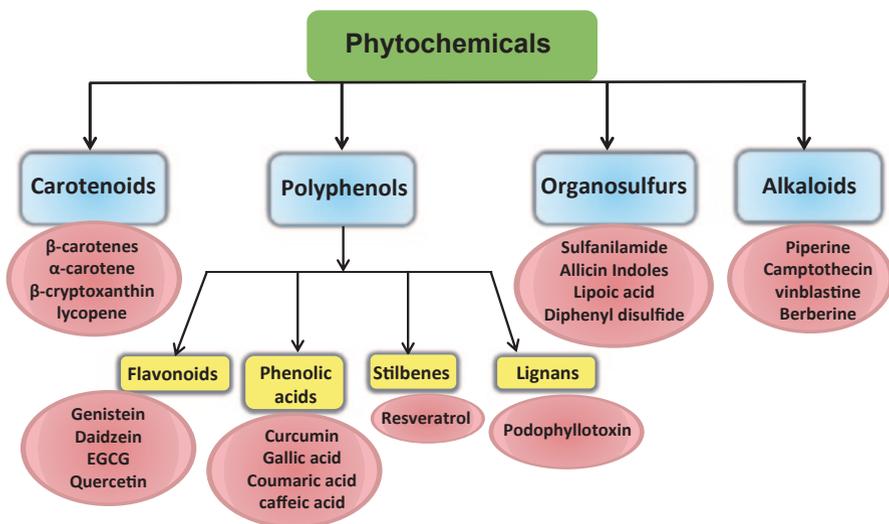


Fig. 1 Classification of phytochemicals based on their chemical structure

stilbenes, hydrolyzable, condensed tannins, lignans, and lignins (Fresco et al. 2006; Liu 2004). Of these, flavonoids and phenolic acids are well studied.

Flavonoids

The flavonoids are a diverse group of phenolic compounds with ketone groups. They are widely distributed in the plant kingdom and more than 6,500 structurally unique flavonoids have been identified in plants (Corradini et al. 2011). Flavonoids can be further subdivided into six different classes based on structural differences in backbone, namely flavonols, flavanones, flavones, isoflavones, flavonols, and anthocyanidins (Middleton E Jr et al. 2000). They are found in high concentrations in fruit skins and play important roles in protection against UV radiation and disease resistance (Bashandy et al. 2009). They are known for their potent antioxidant, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties (Nijveldt et al. 2001). The flavonoids such as quercetin, genistein, kaempferol, catechin, and fisetin possess growth inhibitory activity in various cancers (Knekt et al. 1997; Spagnuolo et al. 2012; Sarkar et al. 2006; Weng and Yen 2012; Fotsis et al. 1998).

Phenolic Acids

Phytochemicals containing a phenolic ring and an organic carboxylic acid group are known as phenolic acids. Phenolic acids are largely known for their antioxidant

potential (Liu 2004). These are further subdivided into two groups, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acid derivatives include p-hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids, which are mainly present in the bound form as a component of tannins and lignins or as sugar derivatives in plant foods. Hydroxycinnamic acid derivatives include p-coumaric, caffeic, curcumin, and ferulic acids. They are commonly present in a complex form with cellulose and lignin (Liu 2004). Curcumin, one of the phenolic acid compounds has received a great deal of attention as a potent cancer-preventing and therapeutic agent (Asher and Spelman 2013). Antiproliferative action of caffeic acid, syringic acid, sinapic acid, protocatechuic acid, ferulic acid, and 3, 4-dihydroxy-phenylacetic acid (PAA) has also been shown in breast cancer (Kampa et al. 2004).

Carotenoids

Carotenoids are defined as the phytochemicals containing a long series of conjugated double bonds (Liu 2004). These are one of the well-known groups of pigments responsible for the red, orange, yellow, and dark green colors of vegetables and fruits (Jacob et al. 2012). Some of the carotenoid derivatives that function as provitamin A are β -carotene, α -carotene, and β -cryptoxanthin. Sources of these carotenoids include carrots, sweet potatoes, winter squash, pumpkin, papaya, mango, cantaloupe, tomatoes, pink grapes, pink guavas, apricots, and watermelons (Liu 2004). Carotenoids play important functions in photosynthesis and photoprotection in plant tissues. Carotenoids, such as astaxanthin, zeaxanthin, and lutein, have received substantial attention because of their provitamin and antioxidant characteristics (Liu 2004; Chan et al. 2009; Bohm et al. 2012; Caperle et al. 1996).

Alkaloids

Alkaloids belong to the highly diverse group of compounds that contain a ring structure and a nitrogen atom (Lu et al. 2012). On the basis of their ring structure, alkaloids can be classified into pyrrolidines, pyridines, tropanes, pyrrolizidines, isoquinolines, indoles, quinolines, and the terpenoids and steroids. These are widely distributed in higher plant families such as Ranunculaceae, Leguminosae, Menispermaceae, and Loganiaceae. Approximately, 3,000 alkaloids have been identified in plants and most of them exhibit significant biological activities. Alkaloids are one of the most active components in natural herbs and some of them have been developed as chemotherapeutics, including camptothecin (CPT) and vinblastine (Lu et al. 2012; Huang et al. 2007). Other alkaloids such as piperine, berberine, matrine, evodiamine, sanguinarine, and tetrandrine possess anticancer activity by modulating multiple signaling pathways causing inhibition of the initiation of cancer, inducing cell cycle arrest, apoptosis, and inhibiting metastasis and angiogenesis (Krishnakumar et al. 2009; Wang et al. 2013; Zhang et al. 2012a, 2012b; Yang et al. 2013; Burgeiro et al. 2013).

Organosulfurs

Organosulfur compounds are characterized by the presence of sulfur. They occur in a large number of medicinal plants including garlic, chinese chive, and welsh onion (Fukushima et al. 1997; Zhang et al. 2013). Organosulfur compounds cause detoxification of carcinogens by altering the activity of cytochromes P450 (CYP) and glutathione S transferases (GST) (Davenport and Wargovich 2005; Tsai et al. 2005). These compounds exert diverse biological effects including carcinogen detoxification, inhibition of cancer cell proliferation, free radical scavenging, and inhibition of DNA adduct formation (Lea 1996; Hassan 2011; Moriarty et al. 2007).

Phytochemicals in Clinical Use or Currently Under Trial as Anticancer Agents

The US National Cancer Institute (NCI) first developed the Cancer Chemotherapy National Service Center in 1955 with an objective to screen natural and synthetic compounds as anticancer agents (Zubrod 1972). With this initiative, paclitaxel isolated from the bark of the Pacific yew tree emerged as the most promising FDA-approved anticancer drug (Vaishampayan et al. 1999). Since then, many plant-derived compounds have been approved by the FDA as chemotherapeutic agents. Some of the significant anticancer phytochemicals are listed in Table 1.

Role of MicroRNAs in Anticancer Properties of Phytochemicals

Due to the growing evidence that miRNAs possess oncogenic or tumor suppressor activities, they are being considered as attractive targets for cancer prevention and therapy. Indeed, several studies have suggested that miRNA targeting serves as one of the important mechanisms in anticancer activities of a variety of bioactive phytochemicals as discussed below:

Modulation of MicroRNAs by Flavonoids

Epigallocatechin-3-gallate (EGCG) is a strong antioxidant flavonoid and possesses significant anticancer activity. It inhibits cancer growth by causing cell cycle arrest and apoptosis (Chen et al. 2004). As one of the mechanisms, it was shown that EGCG induced apoptosis in hepatocellular carcinoma (HCC) by upregulating the expression of miR-16, which, in turn, led to the inhibition of its target antiapoptotic Bcl-2 (Tsang and Kwok 2010). Because Bcl-2 plays a critical role in maintaining mitochondrial integrity, overexpression of miR-16 may lead to mitochondria

Table 1 List of Phytochemicals Used in Cancer Prevention and Therapy

Drug/Chemical	Plant Source	Cancer Types	FDA Approved/Under Clinical Trials	References
Topotecan	Camptotheca acuminata	Ovarian cancer, cervical cancer, small-cell lung cancer, glioma	Approved for cervical, ovarian cancer and lung cancer, glioma-Phase I	(Agelaki et al. 2013; Bruce et al. 2011; Creemers et al. 1996; Musa et al. 2013) http://clinicaltrials.gov/ct/show/
Colchicine	Colchicum autumnale and <i>Gloriosa superba</i> L.	Prostate cancer	Postate cancer-Phase I	http://clinicaltrials.gov/ct/show/
Etoposide and Teniposide	Podophyllum peltatum and Podophyllum emodi	Breast cancer, small-cell lung cancer, acute leukemia	Breast cancer-Phase II, small-cell lung cancer-Phase II	(Bjorkholm 1990) http://clinicaltrials.gov/ct/show/
Taxol, Taxotere (Docetaxel)	Taxus brevifolia Nutt, Taxus baccata	Breast, ovarian, non-small lung, prostate cancer and lymphoid malignancies	Approved for breast, gastric, head and neck cancer, prostate cancer	(Davies et al. 2003; Piccart 2003) http://clinicaltrials.gov/ct/show/
Paclitaxel	Taxus brevifolia	Ovary, breast, lung, bladder, pancreatic, head and neck cancer	Approved for ovarian cancer, breast cancer-Phase II, pancreatic neoplasms-Phase I	(da Rocha et al. 2001) http://clinicaltrials.gov/ct/show/
Berberine	Hydrastis canadensis L., Berberis neeris species and Arcungelisia flav	Lung, liver, prostate, breast cancer and osteosarcoma	Preclinical studies	(Diogo et al. 2011; Vuddanda et al. 2010)
Flavopiridol	Amoora rohituka and Dysoxylum binectariferum	Colorectal cancer, ovarian, pancreatic cancer, lymphocytic leukemia and prostate cancer	Leukaemia-Phase II, Pancreatic cancer-Phase II	(Bible et al. 2012; Dickson et al. 2010; Li et al. 2000; Senderowicz 1999)

Table 1 (continued)

Drug/Chemical	Plant Source	Cancer Types	FDA Approved/Under Clinical Trials	References
Harringtonine and Homoharringtonine	Cephalotaxus harringtonia, C. hainanensis and C. quinensis	Leukemia	Leukemia-Phase II completed	(Shen et al. 2012; Yuan et al. 2011)
Vindesine and Vinorelbine	Catharanthus roseus	Acute lymphocytic leukemia, lung carcinomas, breast cancer, chronic myelogenous leukemia, colorectal cancer.	Non-small cell lung cancer-Phase III trial, breast cancer-Phase II trial	(Cragg and Newman 2005)
Irinotecan	Camptotheca acuminata	Colon cancer, sarcoma	Sarcoma-Phase II Trial	(Fuchs et al. 2006)
Betulinic acid	Betula alba	Malignant brain tumors, ovarian carcinoma, melanoma, leukemia and malignant head and neck squamous cell carcinoma	Melanoma, -Phase II	(Fulda 2009; Mullauer et al. 2010)
Genistein	Genista tinctoria	Prostate cancer	Prostate cancer-Phase II	http://clinicaltrials.gov/ct/show/
Mistletoe	Viscum album L.	Refractory advanced tumor, gastric cancer	Ref Adv tumor-Phase I, gastric cancer-Phase IV completed	http://clinicaltrials.gov/ct/show/
Curcumin	Curcuma longa L.	Colon cancer, pancreatic cancer, cervical neoplasia, and Barretts metaplasia	Colon cancer Phase II	(Bar-Sela et al. 2010)

dysfunction, cytochrome *c* release, and thus apoptosis. In lung cancer, EGCG was shown to upregulate the expression of miR-210, which led to the suppression of the proliferation and anchorage-independent growth (Wang et al. 2011). EGCG also acts as a repressor of androgen receptor (AR) signaling by blocking the expression of AR-regulated miR-21, and thus inhibits prostate cancer cell growth (Siddiqui et al. 2011).

In a recent study, another flavonoid, 3,6-dihydroxyflavone (3,6-DHF), was shown to reverse the enhanced levels of miR-21, while decreasing miR-34a levels in breast carcinoma, which resulted in diminished tumor growth of breast cancer cells through apoptosis induction (Hui et al. 2012). It is also reported that quercetin, which is another plant-derived flavonoid, exerted potent anticarcinogenic effects in colorectal carcinoma cells, when used in combination with resveratrol. This synergistic effect was caused by induction of apoptosis through downregulation of oncogenic miR-27a (Del Follo-Martinez et al. 2013). In another report, genistein treatment was shown to enhance the apoptotic effect of miR-16 in CLL cells (Salerno et al. 2009). In a study on prostate cancer, genistein restored the expression of Apoptosis Ras homologue member I (ARHI), a tumor suppressor gene, by downregulating miR-221 and miR-222 and thus produced anticancer effects (Chen et al. 2011).

Glyceollins, the flavonoids found in nodules of soybean as a response to symbiotic infection, act as antiestrogens that bind to the estrogen receptors and inhibit estrogen-induced tumor growth in breast cancer (Salvo et al. 2006). It was shown that glyceollin treatment of MDA-MB231 cells induced significant upregulation of several tumor suppressor miRNAs such as miR-22, miR-29b, miR-29c, miR-30d, miR-34a, miR-195, miR-181c, and miR-181d. Furthermore, expression of oncogenic miRNAs namely miR-21, miR-193a-5p, miR-185, miR-224, miR-486-5p, and miR-542-5p was significantly decreased following treatment with glyceollins (Rhodes et al. 2012). Some of these miRNAs such as miR-221 and miR-181 have been associated with mitochondria (Sripada et al. 2012), suggesting that phytochemicals also regulate mitochondrial function by microRNAs. Taken together, all these findings demonstrate an important role of miRNAs in anticancer activities of flavonoids.

Modulation of MicroRNA by Phenolic Acids

Curcumin is an active phenol and a constituent of turmeric (*Curcuma longa*). It has been used for many decades as an important component of spice in Indian food and as a traditional medicine in Asian countries (Asher and Spelman 2013). It is shown to have chemopreventive and -therapeutic activities against many tumors (Khor et al. 2006; Sun et al. 2013; Asher and Spelman 2013). Curcumin exerts its therapeutic effects by inhibiting various cancer-signaling pathways and more recently, it has been shown that modulation of miRNAs plays an important role in its anticancer properties (Reuter et al. 2011). In MCF-7 breast cancer cells, curcumin treatment led to the upregulation of tumor suppressor miRNAs, miR-15a

and miR-16, causing induction of apoptosis (Yang et al. 2010). Curcumin upregulated tumor suppressor miR-203 in bladder cancer, which resulted in downregulation of its target oncogenes Akt2 and Src and apoptosis induction, diminished proliferation, migration, and invasion (Saini et al. 2011). In another study, it was shown that curcumin promoted apoptosis in lung cancer by inducing miR-186* expression (Zhang et al. 2010). Furthermore, in colon cancer, curcumin inhibited the transcriptional regulation of oncogenic miR-21 and led to inhibition of growth, invasion, and metastasis, and increased expression of the tumor suppressor Pcd4 (Mudduluru et al. 2011).

Modulation of MicroRNA by Alkaloids

Camptothecin (CPT), which is isolated from the bark of *Camptotheca acuminata* is an effective chemotherapeutic agent against a broad range of tumors (Moertel et al. 1972; Ulukan and Swaan 2002; Zeng et al. 2012). CPT was recently shown to downregulate the expression of miR-125b, which resulted in upregulation of Bak1 and p53, and subsequently induction of apoptosis in human cervical cancer and myelogenous leukemia cells (Zeng et al. 2012).

Modulation of MicroRNA by Stilbenes

The anticancer activity of resveratrol has been shown in a wide array of tumors including breast, prostate, glioma, lung, neuroblastoma, and colon (Bishayee et al. 2010; Athar et al. 2007). Tili and coworkers showed that resveratrol restored the levels of tumor-suppressor miR-663, and it downregulated the expression of several oncogenic miRNAs such as miR-17, miR-21, miR-25, miR-92a-2, miR-103-1, and miR-103-2 in human colon cancer cells (Tili et al. 2010). Hagiwara and coworkers have shown that resveratrol upregulated miR-141 and miR-200c expression in MDA-MB231 breast cancer cells (Hagiwara et al. 2012). Resveratrol-induced upregulation of miR-141 diminished invasiveness, whereas miR-200 resulted in the inhibition of EMT through upregulation of E-cadherin and downregulation of Zeb1 (Hagiwara et al. 2012). Thus, all these studies provide compelling evidence that resveratrol modulates the expression of miRNA signatures in cancer cells to confer its anticancer activity.

Modulation of MicroRNAs by Organosulfurs

Indole-3-carbinol (I3C) present in cruciferous vegetables is converted into metabolically active oligomeric compounds (such as diindolylmethane or DIM) in the stomach (Minich and Bland 2007). Several investigations have suggested that DIM

can modulate the expression of various miRNAs involved in cancer pathogenesis. In breast cancer cells, treatment with DIM inhibited the growth by inducing the expression of miR-21 and degradation of its target Cdc25A (Jin 2011). DIM also induced the expression of members of miR-200 and let-7 families, which were significantly downregulated in gemcitabine-resistant pancreatic cancer cells. This led to the reversal of EMT and decreased chemoresistance (Li et al. 2009). Furthermore, in another study DIM induced the expression of miR-146a and thus inhibited pancreatic cancer cell invasion via downregulation of EGFR, metastasis-associated protein-2 (MTA-2), interleukin-1 receptor-associated kinase 1 (IRAK-1), and NF κ B (Li et al. 2010).

Conclusion

Despite significant progress during the past decades, cancer still remains one of the most diseases affecting millions of people every year. It poses significant health and economic burden not only on the people affected by this devastating disease, but also on society, thus necessitating the need of effective preventive and therapeutic measures. Phytochemicals have proven to be excellent reservoirs of numerous cancer preventive and therapeutic compounds, and a majority of USFDA-approved anticancer drugs for human use are derived from plants. In addition, several investigative studies have identified novel biologically active phytochemicals exhibiting significant potential for development as anticancer drugs. These phytochemicals can affect carcinogenesis at multiple different stages starting from initiation to development and progression and hence can be used as both preventive as well as therapeutic agents. It has also been revealed that phytochemicals confer their anticancer activities by targeting the expression of miRNAs. Aberrant miRNA expression has already been associated with cancer pathogenesis and the ability of phytochemicals to alter their levels is significant in understanding the underlying molecular mechanisms of phytochemical action. Phytochemicals target multiple organelles including mitochondria to exert anticancer activities. Multiple microRNAs are known to localize in mitochondria to regulate cell death and stress response, therefore modulating the levels of mitochondria-localized microRNAs will determine the fate of cancer cells, which may have significance in cancer prevention and therapy. Accumulating data will therefore provide support in translation of investigative phytochemical drugs to clinics. As we make further progress, synthetic or chemical approaches can also be employed to enhance biological activity and bioavailability of phytochemicals to enhance their potency and health benefits, and to combat the dreadful disease of cancer.

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Optical Imaging of Mitochondria for Cancer Therapy

Jonathan F. Lovell

Abstract Mitochondria are essential organelles that provide most of the energy to eukaryotic cells and also regulate programmed cell death. Optical imaging has been used for over 100 years to visualize and characterize these organelles. In recent years, new and improved optical imaging approaches have been used for functional imaging of mitochondria. Notably, several novel small molecule imaging probes have been developed, alongside fluorescent protein probes, to permit optical imaging of mitochondria to shed light on the molecular functioning of the organelle and associated proteins in disease. This chapter examines an historic perspective on some of the major developments in optical imaging of mitochondria.

Keywords Fluorescence imaging • Optical imaging • Mitochondria • Imaging probes

Introduction

Mitochondria are well understood to be the powerhouses of eukaryotic cells and thus play direct or indirect roles of critical importance in all physiological activities. Mitochondria comprise about 10 to 20% of cellular volume and under aerobic conditions power cellular function by generally producing the vast majority (~90%) of the ATP that is generated from glucose oxidation. Mitochondria were first observed more than 170 years ago and began to be well recognized as ubiquitous cellular organelles over 100 years ago (Ernster and Schatz 1981). In the 1950s, knowledge of mitochondria rapidly expanded with the tandem evolution of improved subcellular fractionation methods and the use of transmission electron microscopy. Subcellular fractionation permitted the isolation of mitochondria and elucidation of their chemical composition and rigorous mapping of their complex metabolic pathways. Transmission electron microscopy provided critical

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knowledge of the mitochondria structure, with the mitochondrial outer membrane surrounding the intermembrane space, which in turn surrounds the cristae-forming mitochondrial inner membrane, which in turn surrounds the matrix. In subsequent decades, the nature of mitochondrial structure and the metabolic pathways that occur there, including the Krebs cycle, the electron transport chain, and oxidative phosphorylation have been elucidated. Mitochondria, with their own distinct and relatively stable mitochondrial genome, provide a critical source of genetic information to trace lineage in ways that otherwise would not be possible (Ballard and Whitlock 2004). In addition, mitochondria also play a key role in calcium homeostasis in the cell (Gunter et al. 1994). As an organelle with such critical responsibility, it is no wonder that numerous diseases are correlated with mitochondrial dysfunction. Mitochondrial metabolic imbalances have been implicated in a wide range of diseases including, for example, diabetes (Rolo and Palmeira 2006), Alzheimer's disease (Castellani et al. 2002), Parkinson's disease (Abou-Sleiman et al. 2006), heart disease (Lesnefsky et al. 2001), and cancer (Kroemer 2006). The association of mitochondria with cancer is particularly strong. Warburg famously hypothesized that mitochondrial dysfunction is the "origin of cancer cells" as normal mitochondrial oxidative phosphorylation-based respiration is abandoned in favor of lactate fermentation (Ganapathy et al. 2009). The impairment of normal mitochondria respiration has remained a central issue in tumor biology ever since (Frezza and Gottlieb 2009). The resulting increased rate of glycolysis in cancer cells drives upregulation of glucose transporters, which has led to one of the most widely used cancer diagnostic imaging inventions, ^{18}F -fluorodeoxyglucose, which is a mainstay in clinical positron emission tomography for cancer diagnosis and staging (Kubota 2001).

In addition to the critical metabolic link of mitochondria to cancer, these organelles play an indispensable role in programmed cell death (apoptosis), which is almost invariably misregulated in cancers (Elmore 2007). The mitochondrial intermembrane space (between the inner and outer mitochondrial membrane) contains several protein factors that when released to the cytosol following permeabilization of the mitochondria outer membrane, commit the cell to executing the apoptosis program. It was discovered that cytochrome *c*, which is normally an electron carrier in oxidative phosphorylation, moves into the cytosol to activate caspases during apoptosis. Several other intermembrane space proteins are also active in promoting apoptosis once they reach the cytosol following mitochondrial outer membrane permeabilization. Mitochondrial outer membrane permeabilization represents the final checkpoint for cells to commit irreversibly to apoptosis. The Bcl-2 family of proteins is responsible for regulating the permeabilization of the mitochondrial outer membrane in a complex manner that is still being elucidated (Chipuk and Green 2008). Another chapter in this book, "Mitochondrial Regulation of Cell-Death" by Jäger and Fearnhead is dedicated specifically to this topic.

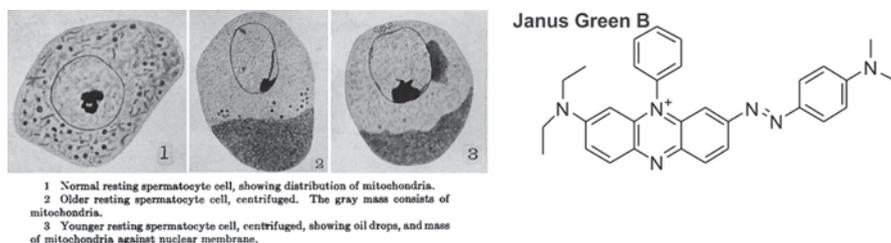


Fig. 1 Optical observation of mitochondria using Janus Green B stain, as reported in 1914. (Browne 1914)

Imaging Mitochondria with Exogenous and Endogenous Probes

Because of the strong link mitochondria have with metabolism and apoptosis, there have been concerted efforts to elucidate better understanding of the organelle structure and function with respect to cancer. Optical imaging was probably the first method used to study mitochondria and has been evolving as a versatile method to understand the roles of this organelle for cancer research. This review covers how optical imaging has helped shape our knowledge of mitochondria and also has a bright future for facilitating new basic and applied approaches for combating cancer.

Exogenous optical imaging probes have been of fundamental importance to develop our understanding of mitochondria (Cottet-Rousselle et al. 2011). One of the most characteristic elements of the inner matrix of the mitochondria is that it maintains a negative electrochemical gradient, creating a mitochondrial transmembrane potential within the matrix, $\Delta\psi_M$, of 220 mV. This is created by the proton pumps of the inner membrane that pump out protons from the matrix into the intermembrane space, where they can freely diffuse through small pores in the mitochondrial outer membrane into the cytosol. The negatively charged, alkaline setting of the mitochondrial matrix provides an environment in which certain contrast agents may preferentially accumulate in healthy mitochondria (that are maintaining their transmembrane potential). Thus, by using cationic amphiphilic dyes that have enough hydrophobicity to retain affinity for membranes, respiring mitochondria may be targeted. As shown in Fig. 1, one of the first methods that was used to identify mitochondria was via Janus Green B staining (Browne 1914). From the figure, which was first published nearly 100 years ago, it is noteworthy that both filaments (“mito”) and grains (“chondria”) are clearly visible within the cell. However, optical contrast staining fell out of favor for studying mitochondria (as did optical imaging in general) for the next 70-odd years as electron microscopy and cellular fractionation became preferred analytical techniques.

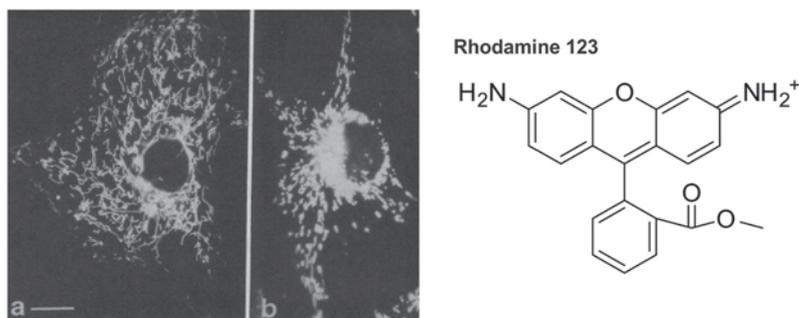


Fig. 2 Rhodamine 123 for live-cell imaging of mitochondria during Rous sarcoma virus transformation. Live Rat-I fibroblast cells transformed with a temperature-sensitive mutant of Rous sarcoma virus and stained with rhodamine 123 at a nonpermissive temperature (**a**) or 30 min after shifting to a permissive temperature. (**b**) Bar represents 25 μm . (Johnson et al. 1980)

It was not until fluorescence microscopy became widely accessible did researchers realize the potential of using small cationic fluorophores for imaging live mitochondria. In particular, the landmark report in 1980 that rhodamine 123 could be used as a versatile probe for imaging live mitochondria supravivally with stunning results revived the field of optical imaging of mitochondria (Johnson et al. 1980). This study demonstrated that rhodamine 123 could be used to image mitochondria in living cells with excellent sensitivity and selectivity. By using rhodamine 123, mitochondria which were typically difficult to observe with phase-contrast light microscopy could be visualized with excellent contrast. As shown in Fig. 2, incubation of living cells with rhodamine 123 permitted fluorescence imaging of mitochondria in exquisite detail. This study directly demonstrated the utility of imaging the mitochondria in cancer by examining changes in mitochondrial morphology following transformation by Rous sarcoma virus. This type of imaging has direct application for determining the role of mitochondria in various cancer-related processes. By extension, this permits new anticancer therapeutics to be evaluated at the molecular level by using mitochondrial imaging methods.

It was subsequently demonstrated that rhodamine 123 exhibits increased uptake into carcinoma cells (Nadakavukaren et al. 1985). This suggested a possible utility of using mitochondrial optical imaging for cancer diagnosis, although this area has remained largely unexplored. Approaches using molecular imaging, including optical molecular imaging, are increasingly being recognized as a valuable strategy for cancer treatment (Weissleder and Pittet 2008). Subsequently, many other small molecule, amphipathic cationic fluorescent probes have emerged for imaging mitochondria (Cottet-Rousselle et al. 2011). These include DiO₆(3), a carbocyanine derivative, as well as JC-1. JC-1 is unique because at high concentrations (when it is taken up into healthy mitochondria), it forms J-aggregates with significantly shifted fluorescence emission properties (Smiley et al. 1991). Thus, by examining different wavelengths which represent the ratio of J-aggregates to monomers, ratiometric

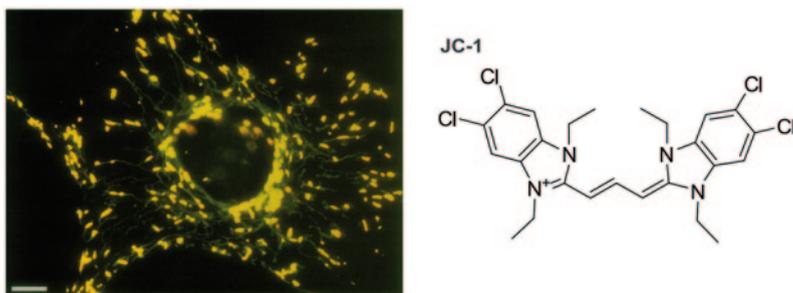


Fig. 3 JC-1 as a ratiometric reporter for the mitochondrial membrane potential. At high membrane potential, JC-1 becomes more concentrated and forms emission-shifted J-aggregates in the membrane. In this micrograph, the heterogeneity of various mitochondria within a single cell is apparent. 8-micron scale bar is shown. (Smiley et al. 1991)

measurements can be performed to assess the state of $\Delta\psi_M$ (Fig. 3). These dyes have been extensively used to investigate how $\Delta\psi_M$ is related to apoptosis and how different chemicals and environments can induce changes in $\Delta\psi_M$ (Ly et al. 2003). One potential drawback of these dyes is that they are not amenable to cell fixation, because $\Delta\psi_M$ is lost during the procedure, thus preventing the use of techniques including immunofluorescence. To address this issue, dyes such as chloromethyl-X-rhosamine (Mitotracker Red) have been created that have a chloromethyl functional group to react covalently with proteins within the mitochondrial inner membrane so that fixation can occur without depleting the mitochondrial distribution of the probe (Macho et al. 1996). Cell fixation, which kills the cells in the process, is required for immunofluorescence to permit reaction of external antibodies with their target to establish localization-specific proteins using fluorescent secondary antibodies. Immunofluorescence has long been used with mitochondria specific antibodies to study diseases in humans (Labro et al. 1978).

As imaging techniques improve, so does the applicability and feasibility of imaging the mitochondria in more complex and relevant systems. Intravital imaging presents some compelling advantages over conventional *in vitro* cell imaging, because the cells have the potential to behave more naturally in their normal environment. The importance of using intravital imaging for more meaningful cancer research in areas such as metastasis is well recognized (Condeelis and Segall 2003). Figure 4 demonstrates intravital imaging of mitochondria in living rats using Rhodamine R6 and two-photon microscopy (Dunn et al. 2002). These approaches open up the possibility of imaging mitochondria in human subjects to assess the organelle properties with respect to cancer therapy or diagnosis. In another example of using emerging imaging practices for mitochondrial imaging, a PET radionuclide chelator was covalently attached to rhodamine for use as a dual modality PET and mitochondrial transmembrane potential optical imaging agent (Zhou et al. 2011). Another new, label-free imaging technique, coherent anti-Stokes Raman scattering, has been able to image lipid rich mitochondria on the basis of their aliphatic nature (Zumbusch et al. 1999).

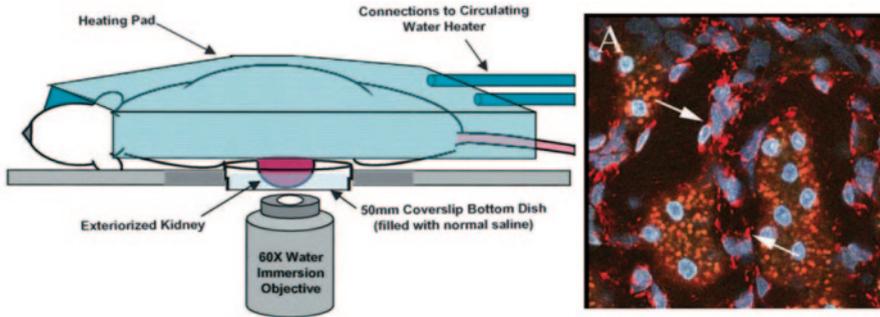


Fig. 4 Intravital optical imaging of mitochondria in living rats. Intravital fluorescence microscopy using a potential-sensitive mitochondrial dye in transgenic rats expressing GFP. Left image shows experimental setup for intravital microscopy. Right image shows micrograph of the kidney of a living rat following intravenous injection of rhodamine R6. Accumulation of rhodamine R6 in mitochondria of endothelial cells is indicated by arrows. Hoechst 33342 stains the nuclei blue. Note that the red fluorescence of rhodamine R6 in endothelial cells can be distinguished from the brown autofluorescent inclusions of the adjacent proximal tubule cells. (Dunn et al. 2002)

Photodynamic therapy (PDT) is a cancer treatment modality that has been adapted in clinical situations for imaging and therapy (Celli et al. 2010). One of the goals of new advanced cancer therapy approaches is to develop rational and feedback-oriented techniques that combine therapy with imaging and diagnosis. Optical contrast agents are uniquely positioned to achieve this because many photosensitizers are able to create both reactive oxygen species for therapy as well as fluorescence for imaging (Lovell et al. 2010). For instance, recent efforts have resulted in developing a sensor that can both induce and monitor apoptosis by using caspase activation as a trigger (Lovell et al. 2011). Many of the small cationic, amphiphilic mitochondrial fluorescent probes depend on active maintenance of a mitochondrial transmembrane gradient therefore they can be used to image and quantify cancer cell death. Twenty-five years ago, rhodamine 123 was used as an indicator dye to quantify the loss in membrane potential during photosensitizer-mediated photodynamic therapy (Singh et al. 1987). In addition to simply reporting on the integrity of mitochondrial transmembrane potential, it was discovered that mitochondria-specific cationic photosensitizers can themselves induce cell death. In a comprehensive study, many mitochondrial photosensitizers were screened for the most effective, potent and selective in cancer cell killing (Oseroff et al. 1986). Certain intramitochondrial sensitizers yielded up to 1000-fold selectivity for cancer cells and functioned at nanomolar concentrations. It was shown that mitochondria are preferred target for the subcellular localization of photosensitizers to achieve maximal cancer cell therapeutic effect (Kessel and Luo 1998). Subsequently, a major research effort focused on the link between the mitochondria and the photosensitizing agent-induced photodamage for anti-cancer therapy (Morgan and Oseroff 2001). Beyond simply using mitochondrial photosensitizers as therapeutic agents, it is also possible to combine therapy with diagnosis in ways that may lead to feedback-oriented theranostic approaches. As

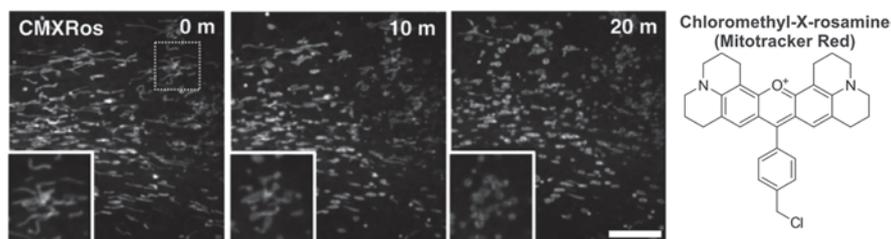


Fig. 5 Using chloromethyl-X-rosamine (CMXRos, also known as Mitotracker Red) to induce apoptosis and detect subsequent morphological changes simultaneously. CMXRos was illuminated to both induce apoptosis in cells and also to induce and detect mitochondrial morphological changes during this process. CMXRos covalently reacts with proteins in the mitochondrial inner membrane, thus it can be used even following loss of mitochondrial membrane potential. Insets show morphology changes. 10 micron scale bar is shown. (Minamikawa et al. 1999)

shown in Fig. 5, chloro-X-rosamine (CMXRos; Mitotracker Red) was demonstrated to photosensitize cancer cells to induce apoptosis, which led to morphological changes that could be detected using the same photosensitizer (Minamikawa et al. 1999). CMXRos, which covalently reacts with mitochondrial inner membrane proteins upon incorporation into respiring mitochondria, is thus retained in the mitochondria even after loss of $\Delta\psi_M$. Unlike mitochondria-localized photosensitizers that require a maintained transmembrane gradient for imaging, CMXRos is suitable for long-term imaging of mitochondria morphology following induction of apoptosis.

It is possible to target conventional optical biosensors to the mitochondria to achieve organelle specific characterization. Optical biosensors are highly diverse and have a variety of activation mechanisms (Lovell and Zheng 2008). Oxidative damage is particularly relevant to the mitochondria, inasmuch as this is the major site of oxidation in the cell and damage can cause mitochondrial DNA mutations, problematic electron transport, loss of calcium homeostasis, and initiation of apoptosis. Robinson et al. demonstrated that by conjugating a hexyl triphenylphosphonium cation to a superoxide optical sensor, selective mitochondrial targeting and detection became possible (Robinson et al. 2006). A similar approach of using a hexyl triphenylphosphonium cation to target mitochondria was subsequently used to create a “light-up” optical sensor for hydrogen peroxide generation in the mitochondria (Dickinson and Chang 2008). As shown in Fig. 6, this sensor localized to the mitochondria and made use of a chemoselective boron-based switching mechanism. Other mitochondria approaches have been used for optical imaging. As shown in Fig. 7 by conjugating a 28-amino-acid peptide sequence to quantum dots, specific mitochondrial localization of nanoparticles was achieved (Deraus et al. 2004). Compared to conventional imaging agents such as CMXRos, quantum dots were resistant to photobleaching and thus could be used to continuously image the mitochondria for extended periods of time. The mitochondrial localization sequence used was taken from cytochrome oxidase subunit VIII (COX 8), which is a protein synthesized in the cytoplasm before being actively transported into the mitochondria. Nanoparticles, with their capacity

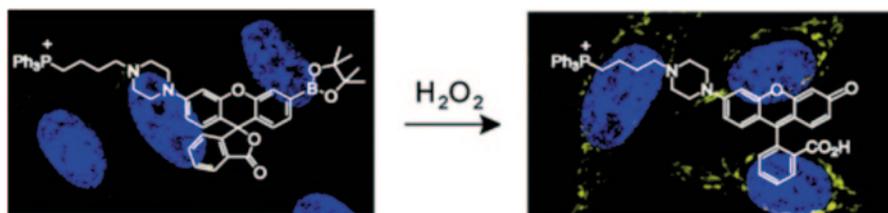


Fig. 6 Developing a mitochondria-specific hydrogen peroxide sensor. A “turn-on” sensor specific for mitochondria created with a hydrogen-peroxide-sensitive moiety as well as a hexyl triphenylphosphonium cation for mitochondria localization. (Dickinson and Chang 2008)

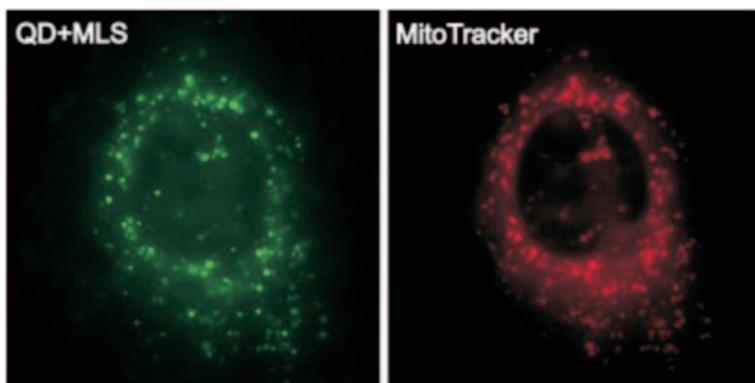


Fig. 7 Targeting nanoparticles to mitochondria using a peptide targeting sequence. A 28-mer mitochondrial targeting peptide was conjugated to quantum dots. Quantum dots localized in mitochondria as judged by colocalization with Mitotracker Red. (Derfus et al. 2004)

to load high payloads of drugs, may prove useful for targeting drugs to mitochondria. Other studies have developed and optimized new synthetic mitochondrial targeting peptides that exhibit excellent mitochondrial localization (Horton et al. 2008). In large part, these developments were based on optical imaging approaches. Subsequently, it was shown that cancer drugs could be rerouted to the mitochondria using the developed peptide (Fonseca et al. 2011).

In addition to exogenous probes, a large body of work has come from imaging mitochondria with proteins tagged with various fluorescent proteins. The emergence of fluorescent proteins in combination with optical imaging and molecular cloning techniques has permitted the visualization and study of proteins and cellular processes in a manner that previously was not possible (Misteli and Spector 1997). In recent years, countless discoveries have been made using fluorescent proteins tagged to mitochondria-related proteins. One of the first examples came when cytochrome *c* was tagged with green fluorescent protein in order to establish that coordinate release of cytochrome *c* during apoptosis is rapid, complete, and kinetically invariant (Goldstein et al. 2000). Figure 8 shows how the cytochrome *c* cellular distribution changes

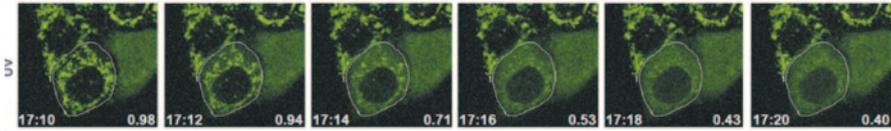


Fig. 8 Monitoring cytochrome *c* release in living cells using fluorescent proteins. Cytochrome *c* release, a major event in apoptosis was monitored by tagging it with GFP and performing optical imaging in live cells. (Goldstein et al. 2000)

during apoptosis from a punctate, mitochondrial pattern to a diffuse intracellular localization. Recently, by using fluorescent proteins, mitochondrial outer membrane permeabilization was visualized using optical imaging *in vivo* (Earley et al. 2012). Such approaches may permit more meaningful evaluation of apoptosis-inducing cancer drugs in the complex tumor microenvironment *in vivo*. In addition to following the movement of proteins, fluorescent protein biosensors have been developed to permit imaging of conditions such as pH within the mitochondria (Kneen et al. 1998).

Conclusion and Future Directions

Mitochondria are intrinsically linked to cancer via both metabolic properties and apoptosis regulation. Optical imaging has been used to study mitochondria for over a hundred years. Small amphiphilic cationic optical contrast agents can selectively accumulate in the mitochondria due to the negative charge within the mitochondrial matrix of respiring mitochondria. Fluorescent probes have revolutionized our understanding of how mitochondria behave. Some photosensitizers can directly destroy cancer cells through light-mediated mitochondrial destruction. New imaging approaches and imaging contrast agents for mitochondria will keep on being developed and used to improve our understanding of cancer behavior and therapeutic responses. These advances will come with the development of both exogenous small molecule probes and endogenous fluorescent proteins. Optical imaging has a both a bright past and bright future for rationally improving the way we diagnose and treat cancer.

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Targeting Cellular Signaling for Cancer Prevention and Therapy by Phytochemicals

Fang Hao, Neelu Yadav and Dhyan Chandra

Abstract Phytochemicals are plant-derived compounds and have been considered as promising chemopreventive agents for decades. Phytochemicals are broadly classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds. These plant-derived compounds modulate the hallmark events in tumorigenesis including the control of cellular redox, cell proliferation and death, angiogenesis, and cell migration. Preclinical studies on a number of phytochemicals in mouse models support anticancer activities of these compounds. Clinical studies of some phytochemicals reveal inconsistency with preclinical findings, thus highlighting the necessity of detailed in vivo analysis of the mechanism of action of these compounds prior to initiate large-scale clinical trials. This chapter describes some of the key molecular cellular signaling that could be targeted to utilize full benefits of these phytochemicals for cancer prevention and therapy.

Keywords Apoptosis • Mitochondria • Autophagy • Phytochemicals • Angiogenesis • Cellular redox • Reactive oxygen species (ROS) • Cell proliferation • Cell migration and invasion • Lycopene • β -Carotene • Clinical trials • Resveratrol • Neem limonoids

Introduction

Phytochemicals are a class of compounds derived from plants and are mostly responsible for the organoleptic properties of plants. Humans consume phytochemicals through consumption of vegetables, fruits, and grains. Early epidemiological studies suggest that higher intake of vegetables and fruits are associated with lower risks for cancers and other chronic diseases. These findings encouraged the cancer research community to search for the components in vegetables and fruits that are responsible for their chemopreventive effects, which prompted studies on a variety of promising candidates in preclinical experimental models and clinical trials. Some of these well-studied components are classified as carotenoids, terpenes, iso-

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flavones, flavonols, stilbenoids, phenolic acids, indoles, glucosinolates, organosulfides, and sterols.

The revolutionary reviews of Hanahan and Weinberg described cancer cells with a series of characteristics (Hanahan and Weinberg 2000, 2011) that include uncontrolled proliferation and cell growth, immortality and resistance to cell death, induction of angiogenesis, occurrence of invasion and metastasis, and development of a protumor microenvironment. The ability of phytochemicals to prevent the occurrence of these changes or reverse the premalignant lesions constitutes the chemopreventive functions of these compounds. A large number of *in vitro* studies indicate that almost all of these cellular processes mentioned above could be modified by phytochemicals, which collectively contribute to their chemopreventive effects observed in *in vitro* studies and animal models. Although the preclinical studies mostly revealed consistent and promising findings, the large-scale epidemiological studies and clinical trials are more controversial. To reconcile such inconsistency, more extensive studies in preclinical animal models are needed to validate the *in vitro* findings and understand the chemopreventive mechanisms of these compounds *in vivo*.

Effects of Phytochemicals on Cellular Redox

Reactive oxygen species (ROS) are important signaling molecules that modulate signaling propagation in cell proliferation and survival (Lenaz 2012; Ray et al. 2012). However, an excessive level of cellular ROS causes oxidative stress that results in damage to macromolecules in cells. Increased oxidative stress is associated with aging and many chronic diseases including cancer. Therefore, production and elimination of ROS are under tight control in order to maintain a healthy reduction/oxidation (redox) balance (Sena and Chandel 2012). Cellular ROS are mainly generated from reactions in the mitochondrial respiratory chain under physiological conditions. Therefore, mitochondrial dysfunction and deregulated metabolism could result in increased ROS production and impaired expression/function of p53, which subsequently contributes to tumorigenesis (Compton et al. 2011; Lee et al. 2011; Wallace 2012).

Most phytochemicals structurally appear as effective antioxidants. For example, lycopene has 11 conjugated double bonds and β -carotene has 9. The extensive system of conjugated double bonds in these compounds gives them the ability to act as antioxidant, singlet oxygen quencher, and free radical scavenger (Burton and Ingold 1984; Di Mascio et al. 1989; Demmig-Adams and Adams 2002; Krinsky and Johnson 2005). These two compounds are among the most common carotenoids and have been closely studied for their health benefits. The main dietary sources for lycopene and β -carotene are tomatoes and carrots, respectively. Both compounds are predominant carotenoids in human plasma but have different patterns of tissue distribution (Bendich and Olson 1989; Nierenberg and Nann 1992).

Lycopene decreases the production of cellular ROS in a dose-dependent manner in prostate cancer cells (Palozza et al. 2010). Similar inhibitory effects on cellular ROS levels were observed in other cancer cell types (Ben-Dor et al. 2005). Consistent with the reported modulatory effects of ROS on Ras/Raf/ERK signaling, phosphorylation, and thus activation of JNK, ERK1/2, and p38 was decreased in parallel with reduction of ROS following lycopene treatment. Nuclear translocation of downstream effectors NF- κ B was also inhibited, which led to the inhibition of cell growth and induction of apoptosis in these cells. The balancing effects of lycopene on cellular redox are also reflected by reduction of cell damage in treated cells. A study found that lycopene alleviates the oxidative DNA damage in hepatoma cells (Park et al. 2005), and it also reduces further damage to DNA and chromosomes induced by ROS such as H₂O₂ in another hepatoma cell line (Scolastici et al. 2008). However, it is likely that the effects of phytochemicals on cellular oxidative stress are dependent on the applied dose and the cell types. Although a lower concentration of lycopene reduces lipid oxidation in prostate cancer cells, higher concentration seems to cause increased oxidative DNA damage in the same type of cells (Hwang and Bowen 2005; Park et al. 2005). Such dose-related inconsistency needs further validation through *in vitro* and *in vivo* studies, which may help determine the recommended dose as dietary supplements for human consumption.

Drug metabolizing enzymes contributing to maintenance of the cellular redox balance and metabolism of carcinogens are also modified by phytochemicals. Drug metabolizing enzymes target xenobiotics at different phases to facilitate their secretion (Sheweita 2000). The roles of these enzymes in activation and deactivation of carcinogens and pharmaceutical agents are of particular interest to cancer researchers. The most common modifications in phase I metabolism are carried out by the cytochrome P450 mixed-function oxidase system. Cytochrome P450 enzymes metabolize xenobiotics into reactive intermediates that could covalently bind to DNA and cause DNA damage. Cytochrome P450 enzymes primarily localize in the microsomes, however, these enzymes are also expressed in mitochondria of human and rodent cells (Sangar et al. 2010). Although cytochrome P450 enzymes share similar functions as their microsomal counterparts, mitochondrial cytochrome P450 enzymes harbor some unique activities (Sangar et al. 2010). Phase I-mediated intermediate compounds are subjected to phase II modifications leading to reduced reactivity and increased polarity. One of the most important enzyme families for phase II modification is the glutathione S-transferase (GST) family, which conjugates the substrates with reductive glutathione. Similar to cytochrome P450 enzymes, mitochondria-specific isoforms of GST are also expressed (Raza 2011). Silencing or knock-out of certain isoforms of mitochondrial GST could result in dysfunction of this organelle.

Consistent with its role as an antioxidant, lycopene increases the expression of certain phase II enzymes in both breast cancer cells and hepatoma cells (Ben-Dor et al. 2005). The enhanced transcription is mediated by transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) and the antioxidant response elements (ARE). Lycopene showed a greater potency in transcriptional activation compared to other carotenoids tested. A number of other classes of phytochemicals, such as

flavonoids, isothiocyanates, and monophenol carnosol also induce phase II metabolizing enzymes through the Nrf2/ARE transcription system (Kong et al. 2001; Martin et al. 2004). Quantitative proteomic analysis also identified a different set of detoxification enzymes whose expression is induced following lycopene treatment in prostate cancer cells (Goo et al. 2007). The functions of detoxifying enzymes are to protect cells from the damaging effects of radicals, hydrogen peroxide, arene, and aliphatic epoxides (Skoda et al. 1988; Yasmineh and Theologides 1993; Michiels et al. 1994). In vitro inducing effects of lycopene on these enzymes are confirmed in vivo, demonstrated by increased enzymatic activity in carcinogen-induced gastric tumors in lycopene-treated mice compared to a control group (Luo and Wu 2011).

In vivo studies found that lycopene also modulates phase I metabolizing enzymes, which contribute to its chemopreventive effects through reduced metabolic activation of carcinogens. Cytochrome P450 isozyme CYP2E1, which is involved in activation of carcinogen diethylnitrosamine (DEN) through demethylation, was modulated by lycopene in vivo (Astorg et al. 1997). This modulation is likely to contribute to the reduced hepatic lesions in mice treated with DEN plus lycopene as compared to DEN alone. A large body of studies found that mitochondrial CYP2E1 could induce oxidative stress and cause cellular damage similar to that of the microsomal form (Sangar et al. 2010), therefore, it will be interesting to investigate the effects of lycopene on mitochondrial CYP2E1 and the biological consequences in further studies. Analysis of rat liver microsomes found that lycopene specifically decreases the activity of nitrosodimethylamine N-demethylase, which is also a phase I metabolizing enzyme, and other carotenoids tested showed different modulatory effects on the drug-metabolizing enzyme system (Gradelet et al. 1996a).

Studies of other phytochemicals in animal models revealed that modulations of phase I and II metabolizing enzymes in favor of enhanced redox balance and carcinogen detoxification are common chemopreventive mechanisms of these compounds. Extracts of the tropical plant neem and Thai bitter gourd, which contains a mixture of bioactive compounds, repressed the phase I modification reactions involved in carcinogen activation and increased the activity of phase II enzyme GST (Kusamran et al. 1998; Dasgupta et al. 2004). Reflective of the alterations of metabolic enzyme activities, the oxidative stress induced by carcinogens DMBA and MNNG is alleviated by treatment with neem extract in mice (Balasenthil et al. 1999; Subapriya et al. 2004). Protein oxidation, lipid oxidation and peroxidation, as indicators of cellular oxidative status, are decreased in liver, blood circulation, and in carcinogen-induced tumors in these mice. The formation of bone marrow micronuclei in response to carcinogens is also decreased by lycopene pretreatment, which suggests reduced genotoxicity in mice treated with both compounds compared to the carcinogen alone (Subapriya et al. 2004). Elevated levels of glutathione in the liver and extrahepatic organs are correlated with increased activity of GST and other phase II metabolizing enzymes induced by neem extract (Dasgupta et al. 2004). The incidence of stomach tumors induced by benzo(a)pyrene and skin papillomas induced by DMBA are significantly reduced in mice treated with neem extract. In addition, tumor burden is also significantly decreased in both mouse models.

Modulation of Proliferation and Cell Growth by Phytochemicals

Uncontrolled proliferation and unlimited growth is one of the most prominent characteristics of cancer cells (Hanahan and Weinberg 2000). Under normal conditions, cell growth is driven by environmental cues, such as growth factors, and successful division into two daughter cells requires sufficient energy to achieve accumulation of biomass and replication of the genome. As the powerhouses of normal cells, mitochondria produce the majority of ATP in the cells through the aerobic respiratory chain. Instead, cancer cells mainly rely on glycolysis even in the presence of sufficient oxygen, which is the well-known “Warburg effect” (Warburg 1956; Chandra and Singh 2011). The reprogramming of normal metabolic pathways allows cancer cells to sustain their uncontrolled growth with a limited supply of oxygen and nutrients in the surrounding environment.

The findings using various cancer cell types suggest that inhibition of the cell cycle and growth are common actions of phytochemicals, demonstrated by decreased cell viability and occurrence of cell cycle arrest. Tomato-derived lycopene induces suppression of proliferation and G0/G1 cell cycle arrest in breast cancer cells and endometrial cancer cells (Ben-Dor et al. 2001; Nahum et al. 2001). Azadirachtin, one of the most-studied bioactive components of neem, induces G0/G1 cell cycle arrest of HeLa cells in a dose-dependent manner (Priyadarsini et al. 2010). Nimbolide, another bioactive component of neem, is also found to inhibit cell cycle progression and growth of HeLa cells, colon carcinoma cells, breast cancer cells, and choriocarcinoma in a dose- and time-dependent manner (Roy et al. 2006; Harish Kumar et al. 2009; Priyadarsini et al. 2010; Elumalai et al. 2012). Analysis of cell cycle distribution revealed that nimbolide induced both G0/G1 and G2/M arrest (Roy et al. 2006). In the case of prostate cancer cells, this antiproliferative effect of neem extract, which is a mixture of a variety of components, does not appear to be affected by the status of androgen dependence of these cells (Gunadharini et al. 2011; Mahapatra et al. 2011). Lycopene similarly inhibits the proliferation of both androgen-dependent and -independent prostate cancer cells (Kim et al. 2002; Hwang and Bowen 2004). It is interesting, however, that hormone-sensitive prostate cancer cells show G2/M phase arrest following lycopene treatment (Hwang and Bowen 2004), whereas hormone refractory cells exhibit G0/G1 phase arrest. The validation of these observations in *in vivo* studies will further determine whether lycopene may provide benefits against recurrent androgen-independent prostate cancer. *In vitro* studies also found that both lycopene (Uppala et al. 2012) and neem extract (Ilio et al. 2006; Ricci et al. 2008) seem to target tumor cells selectively, with little or no growth-inhibitory effect on normal cells.

Key proteins involved in cell cycle machinery such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) are modulated by phytochemicals and other therapeutic agents. Phytochemicals often suppress the expression of cyclins and kinase activity of CDKs in cancer cells while increasing the levels of CKIs expression. Lycopene-induced growth inhibition in breast and endometrial cancer cells involves a decrease of cyclin D1/D3, upregulation of p21, and retention of

p27 in the cyclin E/CDK2 complex (Nahum et al. 2001). In addition to these major components of cell cycle machinery, a variety of other proteins are targeted by phytochemicals for growth inhibition. In addition to involvement of cyclins and CKIs, neem components inhibit cell cycle progression through modulation of proliferating cell nuclear antigen (PCNA) and cell cycle checkpoint proteins CHK2 and Rad17 (Roy et al. 2006; Priyadarsini et al. 2010). Lycopene also regulates structural proteins involved in mitosis, such as β -tubulin, cytokeratin, and heat shock proteins (Uppala et al. 2012).

Cell growth is regulated through a complex network of proproliferative and antiproliferative signaling pathways. The proliferation program is initiated when the cells sense the presence of extracellular growth factors. Therefore, signaling mediated by growth factors, their cognate receptors, and downstream effectors is fundamental to cell growth. Among these pathways, the aberrant activation of insulin-like growth factor 1 (IGF-1) signaling axis in cancer cells appears to be suppressed by lycopene. Physiological concentration of lycopene reduces the phosphorylation of insulin-receptor substrates and increases the cellular level of IGF-binding proteins in breast cancer cells, which collectively lead to weaker IGF signaling (Karas et al. 2000). The alteration of the IGF pathway subsequently affects multiple components of cell cycle machinery, such as retinoblastoma proteins, cyclin D1 and CKIs, and thus delays the cell cycle progression (Karas et al. 2000; Nahum et al. 2006). Similar inhibitory effects on IGF signaling by lycopene are also observed in prostate cancer cells, through modulation of IGF-1 receptors and IGF-binding proteins (Kanagaraj et al. 2007). Since high levels of IGF-1 in circulation have been shown as a risk factor in breast and prostate cancers, the ability of lycopene to repress IGF-1 signaling may be clinically beneficial. Because IGF signaling and cellular ROS mutually regulate each other, it is likely that the growth-inhibitory effects of lycopene are attributed to its modulation on a complex network involving cell signaling and redox balance (Vardatsikos et al. 2009).

Additional classic proproliferative signaling pathways such as the Ras/Raf/ERK and PI3K/AKT pathways are also targeted by phytochemicals to arrest cell cycle progression in cancer cells. Treatment of prostate cancer cells with lycopene suppressed these signaling pathways, which appeared to be independent of the levels of androgen (Palozza et al. 2010). Similar inhibitory effects have been observed in lung cancer cells and colon cancer cells (Tang et al. 2008; Palozza et al. 2010). Subsequently, multiple downstream components of these pathways are affected including NF- κ B, and β -catenin as well as cell cycle machinery proteins (Tang et al. 2008; Palozza et al. 2010). PI3K/PTEN/AKT signaling is often involved in the metabolic switch from oxidative phosphorylation (OXPHOS) to glycolysis in cancer cells, mainly through increased glucose uptake and glycolysis (Jones and Thompson 2009). Therefore, phytochemical-induced suppression of PI3K/PTEN/AKT signaling decreases glycolysis in cancer cells. This is likely to contribute to the proliferation inhibition caused by phytochemical treatment in these cells. Similarly, oncogene c-Myc and transcription factor hypoxia-inducible factor-1 (HIF-1) stimulate the expression of key proteins involved in glucose metabolism (Semenza et al. 1994; Shim et al. 1997; Osthus et al. 2000). Therefore, overexpression of c-Myc

commonly seen in a wide variety of cancer cells and activation of HIF-1 in response to the hypoxic microenvironment of tumors both contribute to increased glycolytic metabolism in cancer cells. Phytochemical dibenzoylmethane (DBM), a β -diketone structural analogue of curcumin, inhibited estradiol-induced proliferation of breast cancer cells as well as induced expression of c-Myc in these cells (Lin et al. 2006). The antiproliferative effects of DBM on mammary cells have also been confirmed in estradiol-treated mice. Topical use or dietary supplement with silibinin, a flavonolignan derived from milk thistle, decreased UVB-induced cell proliferation in mice (Gu et al. 2007). In addition, silibinin also decreased the expression of HIF-1 in the skin tumors developed in UVB-exposed mice (Gu et al. 2007). Although the concomitant inhibition of proliferation and expression of c-Myc and HIF-1 following treatment with phytochemicals suggest a possible causal relationship between these events, further studies are needed to confirm whether reduced expression of these oncogenes directly results in growth inhibition and whether decreased glycolytic metabolism is the underlying mechanism connecting these events. On the other hand, tumor suppressor p53 modulates the metabolic balance in normal cells through upregulation of proteins that are involved in mitochondrial OXPHOS and inhibition of glycolysis (Bensaad et al. 2006; Matoba et al. 2006). Thus, loss of functional p53 in cancer cells further supports the cells in transforming from normal metabolism to increased glycolysis, which allows excessive growth with limited oxygen and nutrients. A study on cervical cancer cells revealed that expression of p53 could be induced by treatment with the flavonoid quercetin and this induction together with NF- κ B inhibition led to cell cycle arrest (Vidya Priyadarsini et al. 2010).

Modulation of Cell Death by Phytochemicals

Induction of apoptosis and other forms of cell death, such as autophagy, is another prominent chemopreventive property of phytochemicals. A long list of cancer cell lines has been tested and induction of apoptosis was consistently seen following treatment with different phytochemicals. There are two separate pathways leading to cell apoptosis: the intrinsic pathway through mitochondria and the extrinsic pathway through membrane-associated death receptors (Galluzzi et al. 2012; Tait and Green 2012). The intrinsic apoptotic cascade is initiated with decreased potential of the mitochondrial membrane, and therefore, increases its permeability to allow the release of apoptogenic factors, such as cytochrome *c*. Released cytochrome *c* then binds to apoptotic protease activating factor 1 (Apaf-1) in the cytosol, which leads to the activation of procaspase-9. Activated caspase-9 then activates downstream effector caspases through protein cleavage and the caspase cascade eventually results in the degradation of multiple key proteins required for cell survival. The release of apoptogenic factors is regulated by the Bcl-2 family of proteins, which includes both proapoptotic members (e.g., Bax and Bak) and antiapoptotic members (e.g., Bcl-2 and Bcl-xL). Therefore, apoptosis through the intrinsic pathway is closely related to mitochondrial functionality and the balance between proapoptotic and an-

tiapoptotic proteins. Thus the members of the Bcl-2 family of proteins are effective targets for phytochemicals in the regulation of apoptosis.

Indeed, multiple types of dietary phytochemicals induce apoptosis in cancer cells. For example, treatment with a physiologically attainable concentration of lycopene reduced mitochondrial transmembrane potential and induced the release of mitochondrial cytochrome *c*, and thus initiating the intrinsic apoptotic pathway in prostate cancer cells (Hantz et al. 2005). The induction of proapoptotic protein Bax and the suppression of antiapoptotic protein Bcl-2 caused by lycopene treatment collectively led to increased apoptosis (Palozza et al. 2010). It is interesting that hormone refractory prostate cancer cells appear more sensitive to the proapoptotic effects of lycopene because a significantly lower concentration of lycopene is required to induce apoptosis in these cells as compared to androgen-dependent prostate cancer cells (Hwang and Bowen 2004; Hantz et al. 2005; Tang et al. 2005; Ivanov et al. 2007). Resveratrol, a natural polyphenol found abundantly in grapes and peanuts, also induces apoptosis through the intrinsic pathway in cancer cells, manifested by reduction of mitochondrial membrane potential, release of cytochrome *c* from mitochondria, and activation of caspase-9 following treatment with resveratrol (Gogada et al. 2011; Lin et al. 2012). Investigation of the underlying mechanisms found that resveratrol induces the interaction between XIAP and Bax in both cytosol and mitochondria, which facilitates the translocation of cytosolic Bax to mitochondria and its homo-oligomerization at mitochondria. Channels formed by Bax oligomers on the outer mitochondrial membrane increase the membrane permeability, which allows the release of cytochrome *c* and activation of caspase-9 (Gogada et al. 2011). Neem limonoids similarly promote the release of cytochrome *c* from mitochondria and modulate the expression of the Bcl-2 family of proteins in a variety of cancer cells (Harish Kumar et al. 2009; Priyadarsini et al. 2010; Elumalai et al. 2012; Srivastava et al. 2012). Note that induction of apoptosis by resveratrol and neem limonoids appear to be p53-independent (Gogada et al. 2011; Srivastava et al. 2012). Since most cancer cells possess either p53 mutation or p53 dysfunction, these phytochemicals may offer extra benefits by inducing apoptosis of cancer cells through mechanisms independent of p53. The apoptosis-inducing effects of neem limonoids also exhibit selectivity towards tumor cells, as they show a significantly higher cytotoxicity towards leukemia cells compared to a normal lymphocyte cell line (Kikuchi et al. 2011).

In the extrinsic pathway, apoptosis is initiated through the binding of death receptors with extracellular cognate ligands, which activates the caspase cascade for apoptosis induction. Expression of and/or interaction between death receptors and cognate ligands are also modified by phytochemicals. In addition to intracellular stress, mitochondria also respond to signaling mediated by an extracellular apoptotic stimulus, which typically initiates extrinsic apoptosis. Activation of the Fas receptor initiates a signaling that leads to changes in mitochondrial membrane permeabilization and the release of apoptogenic factors, including small mitochondria-derived activators of caspases (SMAC) and OMI/HtrA2 (Jost et al. 2009). This interplay points out the possibility that mitochondria could also serve as a hub to connect the intrinsic and extrinsic apoptotic pathways. Mitochondria also release

apoptosis-inducing factor (AIF) in addition to cytochrome *c*, which is involved in caspase-independent apoptosis (Susin et al. 1999). The released AIF eventually translocates to the cell nucleus and induces DNA fragmentation and chromatin condensation (Joza et al. 2001). Release of AIF from the mitochondria by neem limonoids in prostate cancer cells also contributes to caspase-independent cell death (Srivastava et al. 2012).

Apoptosis induction by phytochemicals also shares some common mechanisms with the inhibition of proliferation. For example, lycopene-induced modulation of Ras/RAF/ERK signaling and IGF-1 signaling contributes to its differential effects on both cell growth and apoptosis (Kanagaraj et al. 2007; Palozza et al. 2010). The apoptosis-inducing effects of neem extract are also mediated by NF- κ B signaling demonstrated by the altered expression and activities of proteins involved in NF- κ B signaling including NF- κ B, I κ B, and IKK (Priyadarsini et al. 2010; Schumacher et al. 2011; Kavitha et al. 2012; Manikandan et al. 2012). In addition, since tumor cells are often protected by induced NF- κ B signaling during radiation, neem leaf extract enhances the cytotoxic effects of radiotherapy through suppression of the NF- κ B pathway (Veeraraghavan et al. 2011a; Veeraraghavan et al. 2011b).

Autophagy is another form of programmed cell death and plays an important role in normal cell growth and carcinogenesis (Gozuacik and Kimchi 2004; Mathew and White 2011). Cells that are destined to die through autophagy form so-called autophagic vesicles, which contain intracellular content and require fusion with lysosomal vesicles in cells (Wirawan et al. 2012). Autophagic activity is decreased in transformed cells than in normal cells, and induction of autophagy has been suggested as one of the anticancer mechanism of therapeutic agents. Current evidence supports that autophagy could also account for the cell death induced by anticancer phytochemicals. Treatment with either neem extract or resveratrol induces concomitant autophagy and apoptosis in cancer cells (Prabhu et al. 2012; Srivastava et al. 2012). Mechanistic study revealed that resveratrol triggers autophagy through reduction of mitochondrial DNA content (Prabhu et al. 2012). Therefore, considering the central role of mitochondria in apoptosis, incorporation of damaged mitochondria in autophagic vesicles is likely to contribute to the regulation of apoptosis. Mitochondria also integrate these two forms of cell death (Tait and Green 2012). Beclin 1, a protein that forms a complex with PI3K and promotes autophagy, is identified as a novel tumor suppressor gene with decreased expression in cancer cells (Lin et al. 2013). In resveratrol-treated cancer cells, interaction between Beclin 1 and p53 was detected in both mitochondria and cytosol, which may partially contribute to resveratrol-induced autophagy (Prabhu et al. 2012). The underlying mechanisms are still unclear, however, autophagy and apoptosis are known to crosstalk in different manners. Depending on the context, autophagy may either promote the execution of apoptosis or antagonize it. It is interesting that when neem-induced apoptosis is inhibited by caspase inhibitor, expression of the autophagy marker is further increased following neem treatment in prostate cancer cells (Srivastava et al. 2012). Inhibition of autophagy leads to enhanced caspase activation and apoptosis induced by resveratrol in breast and colon cancer cells (Prabhu et al. 2012). Collectively,

these findings suggest the crosstalk between apoptosis and autophagy regulating programmed cell death.

Phytochemicals Modulate Angiogenesis

Cell growth requires oxygen and nutrients provided by the vasculature in the vicinity. Therefore, development of a more extensive network of vasculature within the tumor mass is necessary to sustain rapid growth of cancer cells. The dependence of tumor growth on angiogenesis has been supported by substantial experimental evidence since it was first proposed by Folkman four decades ago (Sherwood et al. 1971). As one of the hallmarks of cancer, the ability of malignant cells to stimulate angiogenesis is affected by chemopreventive phytochemicals either directly or indirectly. For example, neem extract inhibits tube formation of human umbilical vein endothelial cells (HUVEC) *in vitro*, which is an important event during angiogenesis (Mahapatra et al. 2012). Consistent with the *in vitro* findings, the formation of blood vessels induced by angiogenic factors was also disrupted by treatment with neem extract in mice. This attenuation of angiogenesis is, at least partially, caused by inhibition of the proliferation and migration of HUVEC. Mitochondria with decreased size and fewer cristae as well as morphological changes observed in HUVEC treated with neem extract (Mahapatra et al. 2012) suggest the involvement of mitochondria in the modulation of the angiogenesis process. Evidence also suggests the involvement of mitochondria in antiangiogenic effects of neem extract. For example, mitochondria serve as the powerhouses of the cells and also regulate multiple cellular signaling, which are essential for cell growth and proliferation (McBride et al. 2006; Lee et al. 2011). In addition, stimulation of HUVEC with angiogenic factor VEGF increased mitochondrial metabolism and production of ROS through the mitochondrial respiratory chain, which led to enhanced cell migration (Wang et al. 2011).

Inhibition of angiogenesis by neem components in carcinogen-induced hamster buccal pouch (HBP) carcinogenesis mouse model partially contributes to the reduced incidence of HBP carcinoma (Manikandan et al. 2008; Priyadarsini et al. 2009). Extract of melinjo seeds rich in resveratrol derivatives inhibited the proliferation, migration, and tube formation of HUVEC *in vitro*, and also disrupted angiogenic-factor-induced angiogenesis *in vivo* (Kunimasa et al. 2011). Heyneanol A, a tetramer of resveratrol, exhibited a greater potency than resveratrol in suppressing tumor growth following inoculation with Lewis lung carcinoma cells and decreasing tumor microvessel density (Lee et al. 2006). Similar to those described above, the antiangiogenic actions of heyneanol A and resveratrol also involve inhibition of HUVEC proliferation and capillary differentiation induced by angiogenic factors, likely through preventing the angiogenic factors binding to its receptors. In addition, an independent study found that resveratrol-induced apoptosis in human endothelial cells is stimulated by angiogenic factors (Mousa et al. 2005). This is likely another mechanism of antiangiogenic actions of resveratrol, and presumably

mitochondria could be cellular targets of resveratrol as mitochondria play important roles in the initiation of apoptosis. In addition to direct modulation of endothelial cells, resveratrol may also attenuate angiogenesis through regulation of angiogenic factors. For example, treatment with resveratrol decreased microvessel density in inoculated gliomas, accompanied by decreased VEGF expression in tumor cells *in vitro* (Tseng et al. 2004). Tumor cells are known to provide angiogenic factors to the surrounding microenvironment in order to stimulate angiogenesis, therefore, inhibiting the production of angiogenic factors in tumor cells will lead to attenuation of angiogenesis.

Phytochemicals Target Cell Migration and Invasion

Progression of cancer to an advanced stage is accompanied by the occurrence of metastasis, which involves migration and invasion of tumor cells and creation of a suitable microenvironment at distant sites. Alteration of two classes of proteins are primarily involved during metastasis. One class is adhesion molecules that maintain normal cell–cell contact and cell attachment to the extracellular matrix. Another class is extracellular proteases and related proteins that facilitate the invasion of tumor cells through degrading multiple barriers between the original tumor mass and the distant metastatic site. Studies with colon cancer cells found that lycopene inhibits the migratory ability of these cells through regulation of extracellular proteases and cell adhesion proteins (Lin et al. 2011). Treatment of colon cancer cells with leptin, an adipose-derived hormone, stimulates the migration of these cells (Ratke et al. 2010). However, lycopene inhibits the leptin-induced migration and invasion of colon cancer cells. This process is accompanied by decreased expression of matrix metalloproteinase-7 (MMP-7), upregulation and stabilization of adhesion protein E-cadherin (Lin et al. 2011).

On the cellular level, the metastatic potential of breast cancer cells is related to the abnormality in mitochondrial dynamics involving constant fission and fusion (Zhao et al. 2013). Comparison of metastatic and nonmetastatic breast cancer cells suggests that greater cell invasiveness is correlated with higher levels of mitochondrial fission protein dynamin-related protein 1 (Drp1) and lower levels of mitochondrial fusion protein 1 (Mfn1). Manipulations of the expression of these proteins cause alterations in the morphology and subcellular distribution of mitochondria. In addition, manipulating the levels of Drp1 and/or Mfn1 through molecular techniques results in modulation of the metastatic potential of breast cancer cells. The functional involvement of mitochondria in metastasis is further confirmed by the finding that oxidative stress induced by a disrupted mitochondrial respiratory chain enhances invasiveness of breast cancer cells (Pelicano et al. 2009). Mitochondrial dysfunction and the subsequent increase in ROS production also promote migration of gastric cancer cells (Hung et al. 2012) and hepatoma cells (Chang et al. 2009), suggesting that the regulatory effects of mitochondria on cell migration may be common in a variety of cancer cells.

The development and progression of tumors is heavily depend on the interaction between tumor cells and the surrounding microenvironment. In order to create a fostering environment for tumor growth, cancer cells develop the ability to recruit stromal cells, such as fibroblasts, immune cells, and inflammatory cells. These cells not only provide structural and functional support for localized tumors but are also necessary for metastasis. Similarly, the mobility of stromal cells is also affected by mitochondrial health. Migration and invasion of human fibroblasts seem to be promoted by gene mutations involved in mitochondrial respiratory chain and overproduction of mitochondrial ROS (Taddei et al. 2012). Consistent with the role of mitochondrial dynamics in migration of breast cancer cells described above, the mobility of lymphocytes is also differentially modulated by the fission and fusion of mitochondria (Campello et al. 2006). Whereas mitochondrial fission promotes lymphocyte mobility, mitochondrial fusion shows the opposite effects and suppresses lymphocyte mobility. Studies on human skin fibroblasts suggest that lycopene attenuates the migration of fibroblasts towards melanoma (Wu et al. 2007). Lycopene inhibits fibroblast migration induced by chemoattractant platelet-derived growth factor (PDGF) through physical trapping of PDGF, and thus prevents its binding to the receptors. Further studies are needed to investigate whether modulation of mitochondria are involved in the inhibitory effects of lycopene on cell migration.

Clinical Studies on Phytochemicals and Lessons Learned

Epidemiological studies have unarguably suggested that increased intake of vegetables and fruits is associated with decreased incidence of cancer (Block et al. 1992). These findings lead to extensive research on the chemopreventive effects of various components in vegetables and fruits. Although a number of phytochemicals have been identified as promising chemopreventive agents in preclinical studies, clinical trials are still needed for the majority of potential chemopreventive compounds. One class of the compounds that have been subjected to extensive clinical studies is carotenoids. The association of dietary lycopene with its serum level in different cancer types has been evaluated in epidemiological studies and randomized prevention trials (Seren et al. 2008). A wide variety of cancer types has been under investigation including prostate, lung, gastric, breast, liver, pancreatic, colorectal, bladder, head and neck, and esophageal cancers. Findings from a number of clinical studies suggest that lycopene possesses significant preventive effects against almost all cancer types that have been tested. However, other studies suggest no association between lycopene and cancer incidence. These discrepancies are likely to be caused by multiple factors such as the accuracy of the estimated amount of lycopene intake by the participants in epidemiological studies, variation in the design of the prevention trials, the baseline characteristics of the participants included in the studies, and the interpretation of the obtained data. The findings on β -carotene clinical studies show significant inconsistencies with the chemopreventive properties in preclinical studies. The potential chemopreventive functions of a great number of phyto-

chemicals need to be clinically evaluated, however, caution should be exercised in proceeding from preclinical studies to large-scale clinical studies and a thorough understanding of the acting mechanisms of these compounds is crucial to ensure safety of clinical studies.

β -carotene is a prominent carotenoid and provitamin A found in carrots. The core structure of β -carotene consists of nine fully conjugated double bonds, which make the compound an effective antioxidant, radical scavenger, and singlet oxygen quencher (Burton and Ingold 1984; Demmig-Adams and Adams 2002; Krinsky and Johnson 2005). β -carotene is differentially distributed in a number of tissues, including adipose, skin, intestine, liver, corpus luteum, and eye. Among these, adipose contains the majority of total body β -carotene (Bendich and Olson 1989). The initial observational epidemiological studies found that higher dietary intakes and increased serum levels of β -carotene associate with reduced incidence of certain cancers (Huneharek et al. 2001). Encouraged by these observations and epidemiological studies, large-scale clinical trials were subsequently carried out to confirm the anticancer effects of β -carotene supplementation against various types of cancer. Unexpectedly, most of these trials failed to show any beneficial effect of β -carotene on lowering cancer risks, and some studies even found that pharmacological levels of β -carotene possess adverse effects due to increased incidence of certain types of cancer such as lung cancer (Tanvetyanon and Bepler 2008; Druesne-Pecollo et al. 2010). Further *in vivo* studies in animal models revealed that the doses of β -carotene could be the key determinant for its effects on lung cancer.

Two randomized, double-blinded, placebo-controlled primary prevention trials, the Alpha-Tocopherol, β -carotene Cancer Prevention (ATBC) study and the Carotene and Retinol Efficacy Trial (CARET) study, reveal the disappointing findings that dietary supplementation of β -carotene increases the incidence of lung cancer in high-risk population. The ATBC study from 1985–1993 recruited a total of 29,133 male smokers with age between 50 and 69 years old from southwestern Finland, with the goal to investigate the effects of alpha-tocopherol and β -carotene on lung cancer (Group 1994). The participants were randomly divided into four different groups receiving: (1) alpha-tocopherol (50 mg per day) alone, (2) β -carotene (20 mg per day) alone, (3) both alpha-tocopherol and β -carotene, or (4) placebo. Consistent with previous epidemiological findings, consumption of vegetables and fruits at baseline significantly reduces the risk for lung cancer among participants (Holick et al. 2002). In contrary, supplementation of β -carotene increases the relative risks of lung cancer by 18%, instead of offering protection against lung cancer (The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group 1994). Such increased risk of lung cancer appears to be caused by heavier smoking and more alcohol intake, however, the trend was found statistically nonsignificant (Albanes et al. 1996). An 8% increase of total mortality was also observed, mainly due to more deaths from lung cancer and ischemic heart disease (The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group 1994). Dietary intake and serum concentration of β -carotene at baseline inversely correlate with the number of lung cancer cases (Albanes et al. 1995). However, further analysis confirmed this seemingly protective effects on cancer when correlated with baseline serum concentra-

tion of β -carotene, whereas dietary intake did not correlate with incidence of cancer (Holick et al. 2002). To investigate any possible long-term effects of β -carotene supplementation, a 6-year postintervention follow-up assessment was carried out among 25,563 participants (Virtamo et al. 2003). The adverse effects of β -carotene on lung cancer risk and total mortality both disappeared during the follow-up.

The initial analysis suggests no effect of β -carotene supplementation on other types of cancer (The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group 1994), however, this is not consistent with the findings in the follow-up period. An analysis published in 1998 found 23% higher incidence of prostate cancer in the participants receiving β -carotene than in the placebo group (Heinonen et al. 1998). A later study published in 2009 analyzing the prostate cancer diagnosed at the time found that the mortality rate had no correlation with either β -carotene supplementation or serum concentration of β -carotene at baseline (Watters et al. 2009). In addition, other analyses during follow-up observed no effects of β -carotene on colorectal cancers and gastric cancers (Varis et al. 1998; Albanes et al. 2000; Malila et al. 2002a; Malila et al. 2002b).

The β -carotene and Retinol Efficacy Trial (CARET) conducted in the United States recruited a total of 18,341 individuals with high risks for lung cancer due to a history of smoking or occupational exposure to asbestos (Omenn et al. 1996). Individuals in the active-treatment group received oral administration of β -carotene (30 mg/day) plus retinol (25,000 IU/day) to investigate the effects of the combination of both agents on lung cancer in a high-risk population. Unexpectedly, the trial was stopped 21 months earlier than planned, due to a 28% increase in lung cancer risk and 17% increase in mortality in the active-treatment group compared to the placebo group. No significant effect was observed on incidence of prostate cancer, which has the second highest incidences among other cancers. The chemopreventive effects of higher intakes of vegetables and fruits on lung cancer also disappeared in the active-treatment group and were observed only in the placebo group (Neuhouser et al. 2003).

A subset of individuals in this study, including 278 lung cancer cases and 205 prostate cancer cases and matched cancer-free controls, were included to analyze the correlation between serum concentration of β -carotene and cancer risks (Goodman et al. 2003). Although it was found that individuals with lung cancer or prostate cancer have lower serum concentration of β -carotene than the control group, the correlation is not statistically significant. Postintervention follow-up of 6 years was carried out to evaluate the long-term effects of β -carotene after the oral administration stopped (Goodman et al. 2004). The risks of lung cancer and mortality were still slightly higher in the active-treatment group than the control group, although the differences were not statistically significant. This suggests that the adverse effects of β -carotene were reduced or even diminished without continued supplementation. Interestingly, females appeared to have greater risks for mortality than males during the postintervention period (Goodman et al. 2004).

In an attempt to explain the adverse effects of β -carotene in smokers, a number of factors have been proposed. First, studies found that cigarette smoking reduces the serum concentration of β -carotene (Stryker et al. 1988; Handelman et al. 1996;

Palan et al. 1998; Stram et al. 2002). This raises the question whether this confounding factor explains the chemopreventive effects of β -carotene on lung cancer risks in the initial observational epidemiological studies (Mayne et al. 1996). If the lower concentration of serum β -carotene is caused by smoking, the higher incidences of lung cancer that have been hypothesized to be associated with lower serum β -carotene levels could actually be attributed to cigarette smoking. Similarly, an inverse correlation between alcohol drinking and baseline serum concentrations of β -carotene has been observed (Stich et al. 1986), with the underlying mechanism unclear.

Second, studies in ferrets suggest that the dose of supplemented β -carotene determines whether its effect on the lung is beneficial or harmful. To study the effects of supraphysiological dose of β -carotene supplementation, ferrets received treatment with β -carotene at the dose that is equivalent to the dose used in the CARET study and smoke exposure that was similar to the levels in humans who smoke 1.5 packs of cigarettes per day (Wang et al. 1999). Smoke exposure, when combined with β -carotene supplementation, caused drastic reduction of β -carotene concentration in lung tissue and in circulation. Thus, the unexpected detriment caused by β -carotene supplementation may have multiple reasons. Supplementation of β -carotene significantly causes a decrease in the level of retinoic acid in lung tissue compared to control. Other components in retinoic acid signaling pathways were also differentially modulated following β -carotene supplementation, which collectively led to diminished retinoic acid signaling in these ferrets. The apparent contradiction between these findings and the chemopreventive effects of β -carotene in observational epidemiological studies suggest the possibility that β -carotene may have differential functions at physiological and pharmacological doses. The dose of supplemented β -carotene used in prevention trials are 5–10 times higher than the physiological concentration that could be achieved through dietary consumption. Indeed, supplementation of β -carotene in ferrets at a lower dose that is equivalent to physiological concentration of 6 mg provided protection, although weak, against precancerous lesions in the lung induced by smoke exposure (Liu et al. 2000).

Two other large-scale randomized prevention trials, the Physicians' Health Study (PHS) and Women's Health Study (WHS), have been carried out to study the chemopreventive effects of β -carotene in the general population. The PHS enrolled 22,071 male physicians between 40 and 84 years of age in the United States (Hennekens et al. 1996). Half of the participants were current or former smokers at the beginning of the study in 1982. The participants were randomly assigned to receive 50 mg of β -carotene on alternate days or placebo for 12 years. Surprisingly, the PHS trial found neither beneficial nor harmful effects on incidences of cancer, including lung cancer, or total mortality (Hennekens et al. 1996; Frieling et al. 2000). However, supplementation of β -carotene did cause a modest decrease in incidence of all types of cancer combined among those older than 70 years old or in the highest BMI quartile, or who drank alcohol daily (Cook et al. 2000). In addition, β -carotene had an inverse correlation with prostate cancer cases among the participants in the highest BMI quartile and colon cancer cases among daily alcohol drinkers (Cook et al. 2000).

The WHS tested the effects of β -carotene, aspirin, and vitamin E on the prevention of cancer and cardiovascular disease in 39,876 healthy female participants aged 45 years or older (Lee et al. 1999). The participants randomly assigned to receive supplementation of 50 mg β -carotene every other day showed no significant differences compared to placebo treatment, with respect to the incidences of cancer, cardiovascular disease, and overall mortality. The short duration of this trial could be a factor that affected the outcomes. Although β -carotene supplementation reduces prostate cancer and breast cancer in populations with certain baseline characteristics in a couple of studies (Kirsh et al. 2006; Nagel et al. 2010), a number of additional prospective studies and prevention trials found no protection afforded by this compound against overall cancer incidence (Helzlsouer et al. 1989; Daviglus et al. 1996; Verhoeven et al. 1997; Kirsh et al. 2006; Satia et al. 2009; Nagel et al. 2010; Karppi et al. 2012), or adverse effects on the incidence of lung cancer and prostate cancer (Satia et al. 2009; Karppi et al. 2012).

The overall disappointing results in the prevention trials of β -carotene suggest that dietary supplementation of certain phytochemicals at supraphysiological concentrations may not necessarily provide health benefits. The lessons learned from β -carotene point out the importance of thorough understanding of the agent of interest through studies in animal models before proceeding to clinical trials. Because the ATBC study found that β -carotene supplementation increases the serum concentration of a number of other carotenoids besides β -carotene itself (Albanes et al. 1997), the interaction between different micronutrients needs to be taken into consideration when investigating the chemopreventive effects of dietary supplementation of a single compound. Therefore, the complexity of micronutrients contained in a single kind of vegetable or fruit and the possible interaction between these compounds may also explain the discrepancies between the effects of intakes of healthy food and β -carotene supplementation on cancer incidence. Indeed, although a cohort study of dietary intake of tomato-derived lycopene and risk for colorectal cancer found no significant correlation (Malila et al. 2002a and b), other studies revealed the association between increased consumption of tomato and reduced risk for this cancer (Freudenhiem et al. 1990; Hu et al. 1991; Franceschi et al. 1998). In addition, the dose of the agent recommended for clinical trials needs to be evaluated carefully, as preclinical studies often use a wide range of concentrations that could be significantly higher than the physiologically attainable concentration through dietary intake.

Perspective and Future Directions

The early findings that increased consumption of fruits and vegetables are associated with reduced risks of cancer are consistent and compelling, which point out that certain components in these foods could offer tremendous health benefits. Indeed, *in vitro* studies of the major compounds in various fruits and vegetables have found that most of the compounds of interest could reverse the malignant alterations lead-

ing to tumorigenesis, including but not limited to excessive cellular oxidative stress, unlimited proliferation, evasion of cell death, and abnormalities in angiogenesis and cell mobility. Studies using animal models are limited to a number of most promising phytochemicals based on in vitro findings and are not as extensive as in vitro research. However, the in vivo findings thus far are supportive of the chemopreventive effects of phytochemicals established in in vitro studies and provide physiological relevance to the in vitro data. Therefore, phytochemicals still represent one of the most promising groups of chemopreventive agents, despite the controversial outcomes in the current large-scale epidemiological studies and clinical trials that are inconsistent with the preclinical findings. Characteristics, such as low cost, easy availability, and safety to humans also add to the clinical value of phytochemicals. In order to fully take advantage of their health benefits, more extensive preclinical studies of individual phytochemicals should be carried out. The primary goal is to first identify the candidate phytochemicals that have chemopreventive effects and gain a complete understanding of the underlying mechanisms of their actions. It is also important to determine the appropriate dose of the compounds that is beneficial for humans, as it has been noticed that phytochemicals could have different or even the opposite effects depending on the dose administered. Noteworthy, it is also very likely that the health benefits provided by dietary intake of fruits and vegetables are produced by the combination of a variety of different compounds and their interactions, as certain studies have shown that consumption of whole foods provides greater chemopreventive potency compared to dietary supplement with a single phytochemical compound. Therefore, evaluation of the effects of combination regimens containing multiple phytochemicals is warranted, and combined use of different phytochemicals is also likely to provide increased chemopreventive benefits compared to supplement with a single compound.

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