Inhibition of cancer antioxidant defense by natural compounds

Alicja Sznarkowska¹, Anna Kostecka¹, Katarzyna Meller¹ and Krzysztof Piotr Bielawski¹

¹ Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdansk, Poland

Correspondence to: Alicja Sznarkowska, email: alicja.sznarkowska@biotech.ug.edu.pl

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ABSTRACT

All classic, non-surgical anticancer approaches like chemotherapy, radiotherapy or photodynamic therapy kill cancer cells by inducing severe oxidative stress. Even tough chemo- and radiotherapy are still a gold standard in cancer treatment, the identification of non-toxic compounds that enhance their selectivity, would allow for lowering their doses, reduce side effects and risk of second cancers. Many natural products have the ability to sensitize cancer cells to oxidative stress induced by chemo- and radiotherapy by limiting antioxidant capacity of cancer cells. Blocking antioxidant defense in tumors decreases their ability to balance oxidative insult and results in cell death. Though one should bear in mind that the same natural compound often exerts both anti-oxidant and pro-oxidant properties, depending on concentration used, cell type, exposure time and environmental conditions. Here we present a comprehensive overview of natural products that inhibit major antioxidant defense mechanisms in cancer cells and discuss their potential in clinical application.

INTRODUCTION

Over 60% of currently used antitumor drugs come from natural sources such as plants, fungi and microorganisms. The large scale screening programs for natural products with anticancer activities, e.g., launched in 1950s by Italian research company or in 1960s by the National Cancer Institute (NCI), allowed for identification of bacteria-produced doxorubicin and anaxol (paclitaxel), derived from the bark of the yew tree. Both of these compounds are widely used in chemotherapy regimens in different cancer types. Though their mechanism of action is different as doxorubicin intercalates into DNA and abrogates transcription [1], and anaxol inhibits microtubules depolymerization during mitosis [2], they both induce strong oxidative stress, though by different means [3-5]. Total cellular antioxidant capacity is a known determinant of cancer susceptibility of these drugs [6-8]. Oxidative stress induced by chemotherapeutics is crucial for efficiency, but, on the other hand, contributes to cumulative and irreversible cardiotoxicity observed clinically [9, 10]. These side effects highlight the lack of selectivity of chemotherapy [11]. Therefore, on-toxicatral substances that potentiate action of chemotherapeutics and allow for lowering their concentration are of a particular interest in the anticancer drug field.

ROS IN CELLULAR TRANSFORMATION

Majority of cellular reactive oxygen species (ROS) is produced during aerobic respiration by electron releases from the electron transport chain (ETC) in mitochondria. NADPH oxidase (NOX) is one of the largest contributors to cellular ROS. NOXes catalyze the production of superoxide from O₂⁻ and NAD(P)H [12] (Figure 1). Mitochondrial ROS are produced from superoxide dismutase (SOD) and catalase in the mitochondria. At low concentrations, superoxide production may be involved in cellular signal transduction, but high concentrations of radicals cause oxidative damage to other cellular compounds [16].

Higher steady-state levels of ROS in cancer cells relative to normal cells have been known for around 35 years [17]. Increased ROS are crucial in the initiation of cellular transformation, and ROS play a key role in cancer development. The presence of ROS in cancer cells is thought to be due to the activation of the tumor suppressor gene p53. Increased ROS levels can lead to the formation of DNA damage and mutations, which are then repaired by DNA repair mechanisms. This can result in the accumulation of mutations and the development of cancer. Additionally, ROS can activate the Akt/mTOR signaling pathway, which can promote cell survival and inhibit cell death.

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of carcinogenesis when acquiring new mutations and clonal expansion of initiated cells are needed to establish a tumor. This renders both the cause and the result of cellular transformation: ROS-induced oxidative damage favors production of more radicals and establishes a feed-in loop, increasing mutations rate, activating oncogenes, enhancing metabolic reprogramming and progression of tumors. Then enhanced ROS generation is induced by oncogenic signaling with main drivers: V-Ras, K-Ras, mtp53 and c-Myc [18, 19] and involves both mitochondrial and cytoplasmic ROS. K-Ras-induced cellular transformation was shown to require NOX1 activation through p38/PDPK1/PKCδ/p47phox cascade [20], while expression of Myr-Akt, H-RasG12V and K-RasG12D in murine embryonic fibroblasts (MEFs) conferred increased mitochondrial ROS-dependent soft agar colony formation [21]. Mutations in tumor suppressor genes are often associated with induction of strong oxidative stress and promote survival of cells with high ROS levels. Mutant BRCA1 and p53 were shown to attenuate antioxidant signaling driven by nuclear factor (erythroid-derived 2)-like 2 (Nrf2), contributing to cancer initiation [22, 23].

One of the consequences of excessive damage caused by ROS is changes in mitochondrial membrane permeability resulting in cytochrome C release and apoptotic death [24-29]. To defense, cancer cells boost their antiapoptotic mechanisms like nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway to escape cell death [30, 31]. Decreased mitochondrial activity triggers the glycolytic switch and upregulates glycolytic pathway in order to produce more energy and biomass (ribose, amino acids, fatty acids) for rapidly proliferating cancer cells [32]. Moreover, exposure to oxidative stress induces mutations in mitochondrial DNA as well as in VEGF (Vascular Endothelial Growth Factor) and HIF-1α (Hypoxianducible Factor-1α) genes, promoting angiogenesis and further enhancing metabolic reprogramming of cells [33]. Oxidative stress also changes the tumor microenvironment to support growth.

![Figure 1: Generation and scavenging of reactive oxygen species (ROS).](image)

Electrons released from the mitochondrial electron transport chain (Mito-ETC) and produced by NADPH oxidases (NOX) are the major source of endogenous reactive oxygen species. Coupled to molecular oxygen they give rise to the primary free radical and the precursor of remaining species - superoxide (O₂⁻). In the reaction with a short-lived nitric oxide (NO), superoxide forms a highly reactive peroxynitrite (ONOO⁻) able to modify structure and function of proteins. Alternatively, superoxide dismutase (SOD) converts superoxide to hydrogen peroxide (H₂O₂), which can be further transformed in several ways. In the presence of transition metal ions like Fe²⁺ (Fenton’s reaction) or in reaction with superoxide, H₂O₂ forms highly reactive hydroxyl radical (OH⁻) which damages lipids, proteins and DNA. Peroxosomal enzyme catalase (CAT) neutralizes H₂O₂ to water and oxygen. H₂O₂ might be also utilized in the reaction of oxidation of monomeric glutathione (GSH) to the glutathione disulfide (GSSG) or reduced thioredoxin (Trx) catalyzed by glutathione peroxidase (GPX) or peroxidases involved in the thioredoxin turnover (PRX). Reduced glutathione pool is restored by glutathione reductase (GR) which reduces oxidized glutathione with the use of NADPH. Similarly, thioredoxin reductase (TrxR) balances the amount of reduced Trx by transferring electrons from NADPH to oxidized catalytic sites. Thanks to the thiol groups in the Cys residues both glutathione and thioredoxin participate in the reduction of oxidized proteins. Their synthesis as well as the turnover are under tight homeostatic control creating a system responsible for reduction of redox-sensitive proteins upon oxidative stress.
and cell spread. Hydrogen peroxide produced by tumor tissue can initiate destruction of non-tumor surrounding tissue and promote growth [34]. This explains why tumors are said to be “addicted to ROS signaling”.

**ROS ADAPTATIONS IN TUMORS**

Dissimilating homeostasis and higher intracellular ROS levels in cancer cells drive their growth and metastasis but might also pose a threat to oxidative damage and death. Moderate expression of NADPH oxidase NOX5-L induced cancer cell proliferation accompanied by AKT and ERK phosphorylation, whereas an increase in NOX5-L above a certain threshold promoted apoptosis [35]. Tumors adapt to oxidative stress conditions and do so by enhancing their antioxidative defense and lowering ROS levels and by inducing autophagy to protect oxidative damage to biomolecules and organelles [36-39]. These mechanisms constitute finely orchestrated and interconnected repair systems in oxidatively stressed cells seeking homeostasis [36]. Interestingly, the same oncogene signals that boost ROS signaling, promote antioxidant adaptive mechanisms, and help tumors adapt to oxidative stress and minimize oxidative damage. Activation of endogenous K-Ras(G12D), B-Raf(V619E) and Myc(ERT2) led to lowering of intracellular ROS due to increased transcription of Nrf2 and elevation of basal Nrf2 antioxidant programs [40]. Furthermore, genetic targeting of the Nrf2 pathway impaired K-Ras(G12D)-induced proliferation and tumorigenesis in vivo, pointing to the Nrf2 pathway as a previously unappreciated mediator of oncogenesis [40]. Accordingly, it was reported that

**Figure 2: Regulation of Keap1-Nrf2 pathway.** Under basal conditions, cytosolic repressor Kelch-like ECH-associated protein 1 (Keap1), a substrate adaptor protein for Cullin 3 (Cul3)/Rbx1 ubiquitin ligase, holds Nrf2 in the cytoplasm and promotes its ubiquitination followed by 26S proteasomal degradation [58,59]. In the presence of electrophilic and/or oxidative stimulus, Nrf2 is released from Keap1 and translocates to the nucleus where it recruits small Maf protein (sMaf) and binds with response element (ARE) in the promoter regions of its target genes, inducing their expression. Activation of Nrf2 pathway allows for cell adaptation and survival by regulating expression of antioxidants, anti-inflammatory and phase II detoxification enzymes such as superoxide dismutase (SOD), gluthatione S-transferase (GST), heme oxygenase-1 (HO-1), NAD(P)H-quinone oxidoreductase (NQO1), UDP-glucuronosyl transferases (UGT), γ-glutamylcysteine synthetase (γGCS) and efflux pumps like multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP). Proteins transcriptionally controlled by Nrf2 take part in biosynthesis, utilization and regeneration of glutathione, thioredoxin, and NADPH resulting in restoration of cellular redox homeostasis.
genetic mutations that occur in cancer cells lead to constant Nrf2 activity and enhanced antioxidant capacity [41]. Harris et al. (2015) showed that synthesis of the antioxidant glutathione (GSH) was required for cancer initiation in vivo [42]. Genetic loss of the enzyme driving GSH synthesis, glutamate-cysteine ligase modifier subunit (GCLM), prevented tumor’s ability to drive malignant transformation. Interestingly, at later stages of tumor progression GSH became dispensable potentially due to compensation from an alternative antioxidant pathway - the thioredoxin pathway, demonstrating the importance of GSH and thioredoxin in tumor progression and indicating them as potential targets for therapeutic intervention.

Mitochondrial ROS are major inducers of autophagy, however, upon chronic impairment of mitochondrial function, high levels of radicals shifts signaling into self-removal of mitochondria through a selective process called mitophagy [43, 44]. This fine mechanism allows autophagy to eliminate the source of oxidative stress and protect the cell from oxidative damage.

Recently, autophagy was shown to prevent the initiation of hepatocarcinogenesis and metastasis of gastric cancer by maintaining healthy mitochondria and reducing oxidative stress and DNA damage [45-47]. On the other hand, once cellular transformation was initiated, autophagy was required to promote cancer progression by limiting tumor suppressors [45].

**TARGETING ROS ADAPTATIONS IN CANCER**

Because of its sharp reliance on ROS production, cancer cells are more vulnerable to further disturbance of their redox status than normal cells. This difference establishes a therapeutic window allowing for the emergence of the selective anticancer strategy based on modulation of cancer cells’ redox potential. Due to enhanced antioxidant capacity of tumors, just inducing ROS generation is not sufficient for a successful eradication of cancer. The drug...
| BIOACTIVE COMPOUND | TYPE | SOURCE | MECHANISM OF ACTION |
|---------------------|------|--------|---------------------|
| Apigenin            | Polyphenol | Fruits and vegetables | Reduces Nrf2 expression through down-regulation of PI3K/Akt pathway [67] Sensitizes tumor xenografts to doxorubicin [67] Induces glutathione depletion [94] and inhibits mitochondrial complex I activity in rats [151] |
| Chaetocin           | Polyphenol | Chaetomium spp. fungi | Inhibits TrxR in vitro [117]; induces oxidative stress-mediated death of myeloma [152] and glioma cells [118] |
| Chrysin             | Polyphenol | Passion flowers, chamomile, honeycombs, oyster mushrooms | Reduces Nrf2 expression in hepatocellular carcinoma through down-regulation of PI3K-Akt and ERK pathways re-sensitizing cells to doxorubicin [153] Depletes glutathione and enhances doxorubicin-induced cytotoxicity in epithelial cancer cells [69,94] |
| Curcumin            | Polyphenol | Rhizomes of Curcuma longa | Inhibits TrxR required for curcumin-induced radiosensitization [107,119] Inhibits NFκB signaling in different cancer types [154–157] |
| Epigallocatechin gallate (EGCG) | Polyphenol | White, green and black tea (buds and leaves of Camellia sinensis) | Inhibits TrxR and induces cancer cells death [158] Inhibits catalase, leads to elevated ROS [129] Degrades catalase via JNK in endothelial cells [159] Synergize with luteolin to induce apoptosis and p53 activation in cancer cells, reducing growth of xenografts [113] |
| Luteolin            | Polyphenol | Celery, green pepper, parsley, perilla leaf, and chamomile tea | Reduces Nrf2 expression in non-small-cell lung cancer cells, leading to GSH depletion [160] Sensitizes cells to oxaliplatin, bleomycin, doxorubicin [160,161]. Re-sensitizes oxaliplatin-resistant colorectal cancer cells [68] Inhibits Nrf2 in xenografts [162] |
| Myricetin           | Polyphenol | Citrus spp. | Blocks GST activity in melanoma cells [160] Inhibits TrxR leading to death of lung carcinomas [114] |
| Quercetin           | Polyphenol | Citrus spp. | Inhibits TrxR leading to death of lung carcinomas [114] Inhibits mitochondrial complex I activity in rats [151] |
| Resveratrol         | Polyphenol | grapes, raspberries, blueberries, mulberries | Directly binds and inhibits NQO2 and GSTP1 [163–165] Blocks mitochondrial I and III complex activity in colon cancer [166] |
| Wogonin             | Polyphenol | roots of Scutellaria baicalensis Georgi | Down-regulates Nrf2 in resistant myelogenous leukemia cells by modulating PI3K/Akt and DNA-PKcs [167] Inhibits catalase, increasing H2O2. Synergistically sensitizes cancer cells derived from cervix, ovary and lung to TNF-induced apoptosis by blocking TNF-induced NF-kB activation [127] |
| Brusatol            | Alkaloid | Brucea javanica | Reduces Nrf2 via Nrf2 ubiquitination and degradation [73] Sensitizes xenografts to cisplatin via Nrf2 inhibition [73] Down-regulates Nrf2, leading to ROS accumulation. Sensitizes mammospheres to taxol and reduces the anchorage-independent growth [75] Inhibits Nrf2 in freshly isolated primary human hepatocytes [74] Enhances efficacy of cisplatin [64] |
| Piperlongumine      | Alkaloid | Fruits and roots of long pepper | Binds GSH and inhibits its metabolism in leukemias [92] Increases IxBα and suppresses NFkB in human gliomas resulting in ROS-induced apoptosis [93] |
| Trigonelline        | Alkaloid | coffee | Reduces nuclear accumulation of Nrf2 in pancreatic cancer cells and sensitizes them to anticancer drugs and TRAIL via Nrf2 inhibition [76]. Enhances response to chemotherapy in vivo [76] |
should also inhibit the antioxidant defense system [48]. Many compounds of natural origin block Nrf2 pathway or directly inhibit endogenous antioxidants leading to elevated ROS production. Moreover, Nrf2 inhibition results in a decrease of drug efflux transporters and a consequent increase in retention of anticancer drugs in cells. Therefore Nrf2 or cellular antioxidant inhibitors synergize with classic chemotherapeutics and decrease toxicity. Surprisingly, among these compounds are polyphenols like resveratrol, quercetin, EGCG, apigenin, luteolin or chrysin which were initially reported to have ROS scavenging properties and are generally recognized as antioxidants. Therefore a considerable caution should be exercised when applying natural products as adjuvants since their effects strongly depend on concentration, cell type, exposure time and environmental conditions [49-55].

**THE NRF2 PATHWAY**

Disruption of redox balance in cells results in activation of redox-sensitive transcription factors like Nrf2, NFkB and activator protein 1 (AP-1) [56]. The major driver of antioxidant expression is Nrf2 transactivation factor [41, 57]. Activation of Nrf2 pathway allows for cell adaptation and survival by upregulating expression of antioxidants, anti-inflammatory and phase I detoxification enzymes (Figure 2). Major regulator of Nrf2 activity in cells is the cytosolic inhibitor Keap1, responsible for its ubiquitination and proteasomal degradation [58, 59]. Apart from Keap1, oncogenes like K-Ras(G12D), B-Raf(V619E) and Myc(ERT2) have been shown to stabilize Nrf2 and antioxidant proteins leading to drug resistance in tumors [40]. Nrf2 is overexpressed in several types of human cancer, including cancer of the lung, breast, esophagus, ovary, prostate, pancreas, colorectal, head and neck squamous cell carcinoma, gallbladder and skin which indicates that the cytoprotective properties of the Nrf2 pathway can be exploited by tumor cells to promote their survival [60, 61]. Constitutive Nrf2 activation has been shown to mediate chemoresistance in many tumor types [62, 63]. Suppression of Nrf2 activity inhibited tumor growth and enhanced the efficacy of chemotherapeutic agents. Disruption of Nrf2 pathway in a mouse model of K-RasG12D-induced lung cancer enhanced the antitumor efficacy of cisplatin [64]. Temporal blockage of Nrf2-dependent cytoprotection using Nrf2 inhibitors is important and can improve the efficacy of chemotherapy. Nrf2 pathway is a double-edged sword: activating this pathway is crucial for chemoprevention but once control is lost, it provides growth advantage to cancer cells allowing for rapid proliferation, escape from apoptosis or senescence and resistance to chemotherapeutic or radiotherapy. Thus,
both activation and inhibition of Nrf2 activity could be beneficial, although in different patient cohorts (Figure 3).

**NATURAL PRODUCTS TARGETING NRF2 PATHWAY**

Natural product-derived inhibitors of Nrf2 pathway induce ROS insult in ROS-sensitive cancer cells which might result in cell death. Importantly, they often sensitize canceroskeletal effects of chemotherapeutics oradiotherapy through the down-regulation of detoxificationenzymes and drugtransporter enzymes [65, 66]. A significant group of Nrf2 inhibitors belongso polyphenols (see Table 1). Polyphenols are generally recognized as antioxidants and anti-inflammatory agents. At lowo micromolar concentrations polyphenols as quercetin, EGCG, eseretol or curcumin inhibit antioxidant and chemopreventive properties. They can scavenge free radicals either directly, due to presence of OH groups donating a hydrogen atom to a free radical, or by indirect action through the induction of Nrf2 pathway or inhibition of ROS generation. Higher doses of polyphenols (>50 µM) and a presence of transition metal ions promote their pro-oxidant actions like suppression of antioxidant systems and inhibition of Nrf2 pathway [49]. Antitumor effects of flavones like apigenin, chrysin, luteolin and wogonin waselated to downregulation of Nrf2 expression mainly by disturbing PI3K/Akt pathway in cancer cells and in vivo mouse models. Nrf2 inhibition sensitized cancer cells to classic chemotherapeutic drugs like doxorubicin, oxaliplatin or paclitaxel both in vitro and in vivo studies [67-70]. Interestingly, also opposite activity of apigenin, luteolin and chrysin waseported in primary hepatocytes and skinidermal JB6 P+ cells. These flavones induced Nrf2/ARE response and protected against oxidative stress [71, 72]. Differences in their activity between normal and cancer cells and encourage further investigation of their potential in in vivo studies and clinical trials. So far, one of these flavones has been tested clinically for the potential of cancer activity in combination with chemotherapy. Brusatol, aiterpenoid from *Brueca javanica* - an evergreen shrub grown in Southeast Asia and Northern Australia, was described to inhibit Nrf2 signaling by enhancing ubiquitination and subsequent degradation of Nrf2 in different cancer cell lines and mouse xenograft models [73]. Brusatol sensitizes murroscisplatin andaxol [73-75]. The bitter coffee alkaloid, rigeonelline, inhibited nuclear accumulation of Nrf2 in pancreatic cancer cells (Panc1, Colo357 and MiaPaca2) and H6c7 pancreatic duct cells and enhanced their sensitivity to anticancer drugs and TRAIL-induced apoptosis [76]. Aaphthoquinone derived from *Plumbago* species, plumbagin, inhibited nuclearexpression of Nrf2 in humanonts squamous cell carcinoma cells which suppressed expression of Nrf2 downstream target genes resulting in inhibition ofpidermal mesenchymal transition (EMT) and stemness [77]. Parthenolide, a sesquiterpene lactone found in feverfew products, was recently reported to inhibit Nrf2 protein level in breast cancer stem-like cells, derived from dissociation of mammospheres which correlated with an increased ROS production and ledoecrosis [78].

**THE CELLULAR ANTIOXIDANT DEFENSE**

Increased levels of free radicals enable tumor cells to activate pathways driving proliferation, angiogenesis, metastasis and drive under hypoxic conditions [79-81]. High levels of ROS create a risk of damage linked to oxidative stress, therefore cancer cells tend to overexpress detoxifying proteins that activate an antioxidant capacity. Hyper-activation of Nrf2 pathway increases the amount of cellular ROS scavengers. Lowering stress burden by means of enhancing detoxifying force further affects certain pathways promoting growth and proliferation [82-84]. Blocking antioxidant activity in cancer cells decreases their ability to balance oxidative insult and might result in cell death [85]. Below are presented key cellular antioxidant systems and natural compounds disturbing their activity.

**GSH**

One of the major systems involved in the production of free radicals, glutathione. The sulphhydryl (SH) group of glutathione accounts for its electron-donating properties (Figure 1). Once oxidized, it is converted into its dimer by a disulfide bridge (GSSG). GSH is effective in its redox forms and protects them from further oxidation. Glutathione can only directly scavenge free radicals (hydroxylradical, singlet oxygen), but also serves as a cofactor of several detoxifying enzymes like glutathione peroxidase, glutathione reductase. GSH is involved in the metabolism of antioxidants, including vitamins C and E [86]. Most of cellular GSH content remains in cytosol, however it can also be found in organelles, including mitochondria, peroxisomes, and the endoplasmic reticulum [87]. Given their prominence in keeping cells' redox homeostasis in check, glutathione metabolism is accelerated in many types of cancer to alleviate oxidative stress and promote proliferation and metastasis [88]. High levels of GSH are associated with apoptosis-resistant phenotypes and its depletion is linked to a higher risk of cell death initiation [89]. Nuclear and mitochondrial pool of glutathione plays an important role in protecting DNA from oxidative stress-driven lesions. Cell death induced by an intercalating drug doxorubicin was potentiated upon glutathione depletion [89]. This might serve as an alternative therapeutic effect for anticancer agents.
Table 2: Representative clinical trials on natural compounds modifying antioxidant response (from www.clinicaltrials.gov)

| Compound/dose | Clinical trial number/phase | Purpose | Results/Status |
|---------------|-----------------------------|---------|---------------|
| EGCG 40 to 440 µmol/l 3 times a day in combination with etoposide, cisplatin, and radiotherapy | NCT01481818 Phase I | To evaluate safety and efficiency of EGCG in esophagus protection in patients with locally advanced stage III non-small-cell lung cancer | No dose-limiting toxicity of EGCG was reported. Regression of esophagitis to grade 0/1 was observed in 22 of 24 patients at the end of radiotherapy. The pain score was reduced [143] |
| EGCG 40 to 660 µmol/l spray in the radiation field | NCT01481818 Phase I | To assess safety, tolerability and preliminary effectiveness of topical EGCG for radiation dermatitis in patients with breast cancer receiving adjuvant radiotherapy | The topical administration of EGCG was well tolerated and the maximum tolerated dose was not found. Patient-reported symptom scores were significantly decreased at 2 weeks after the end of radiotherapy in pain, burning, itching and tenderness [144] |
| EGCG 10 ml solution/day (440 µmol/l) | NCT02577393 Phase II | To evaluate the protection of the esophagus from damage induced by radiotherapy in patients with lung cancer | enrolling participants |
| Polyphenon E (PolyE, a defined green tea polyphenol extract with high EGCG content) 4 x 200 mg/day | NCT00676793 Phase II | To evaluate the short-term effects of PolyE administered during the interval between breast biopsy and surgery in women with recently diagnosed breast cancer: determination if EGCG inhibits c-Met signaling and activation of pathways contributing to breast cancer progression | completed, no results published |
| Polyphenon E, 4 x 200 mg/day | NCT00676780 Phase II | To evaluate the short-term effects of PolyE administered during the interval between prostate biopsy and radical prostatectomy in men with recently diagnosed prostate cancer | A significant reduction in serum levels of prostate-specific antigen (PSA), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) was observed [181] |
| Polyphenon E, 2 x 200 mg/day | NCT00596011 Phase II | To determine if PolyE reduces the rate of progression to prostate cancer (PCa) in men diagnosed with high-grade prostatic intraepithelial neoplasia (HGPIN) or atypical small acinar proliferation (ASAP) | No differences in the number of prostate cancer (PCa) cases were observed but there was a decrease in a cumulative rate of progression to PCa or ASAP in a PolyE group vs. placebo group [182] |
| curcumin, 6 g/day during radiotherapy | NCT01246973 Phase II/III | To determine whether curcumin can prevent or reduce the severity of dermatitis caused by radiation therapy in breast cancer patients | Curcumin reduced the severity of radiation dermatitis in breast cancer patients [145]. |
| curcumin 2 or 4 g/day for 30 days | NCT00365209 Phase IIa | To evaluate how well curcumin works in preventing colon cancer in smokers with aberrant crypt foci (ACF) | A significant 40% reduction in ACF number was observed with the 4 g dose, whereas in the 2 g group ACF were not reduced. Curcumin was well tolerated at both doses [146] |
| nanostructured lipid curcumin particle 2 x 100 mg/day | NCT02439385 Phase II | To evaluate progression-free survival in colorectal cancer patients with unresectable metastasis after treatment with Avastin/FOLFIRI in combination with a nanostructured lipid curcumin particle which improved biotransformation and bioavailability of curcumin. | This study is not yet open for participant recruitment. |
| Meriva (lecithinized curcumin delivery system) 2 x 500 mg/day | NCT01740323 Phase II | To determine if curcumin reduces NF-κB DNA binding in patients receiving radiotherapy for their breast cancer after having completed chemotherapy | This study is currently recruiting participants |
Piperlongumine (PL), an alkaloid derived from long pepper was described to induce ROS in cancer but not in normal cells [92, 93]. Further studies revealed that PL treatment led to a depletion of cellular GSH and promoted ROS. The activity of chrysin and apigenin towards GSH was assessed in numerous cancer cell lines, including prostate (PC-3), myeloid (HL-60) and lung (A549) cells. Both flavones proved to be effective glutathione depleting agents. Additionally, chrysin potentiated curcumin cytotoxic effect in PC-3 and HL-60 cells [94]. Doxorubicin and cisplatin cytotoxicity was also strongly induced upon chrysin treatment, which promoted GSH efflux and depletion [69, 94]. Another flavone luteolin attenuated Nrf2 signaling leading to decreased expression of its target genes and GSH depletion in wildtype mouse small intestinal cells. Luteolin sensitized oxaliplatin-resistant colorectal cancer cell lines to oxaliplatin, doxorubicin and oxaliplatin [68] and efficiently inhibited GST leading to GSH depletion in melanoma cells [95].

### NATURAL PRODUCTS DISTURBING GSH METABOLISM

| Natural Product | Dose | Study Details | Recruitment Status | Notes |
|-----------------|------|---------------|-------------------|-------|
| **Curcumin**    | 8 g/day along the chemotherapeutic protocol of weekly gemcitabine | NCT00192842 Phase II | To assess if curcumin can improve the efficacy of the standard chemotherapy gemcitabine in patients with advanced pancreatic cancer. | 5 out of 17 patients (29%) discontinued curcumin due to intractable abdominal fullness or pain, and the dose of curcumin was reduced to 4 mg/day because of abdominal complaints in 2 other patients. One of 11 evaluable patients (9%) had partial response, 4 (36%) had stable disease, and 6 (55%) had tumor progression. [183] |
| **Curcumin**    | dosage not provided | NCT02095717 Phase II | To assess taxotere plus curcumin combination in first-line treatment of prostate cancer metastatic castration resistant. | Study is ongoing |
| **NanoCurcumin** | SinaCurcumin® 3 x 40 mg/day 3 days before and during radiotherapy | NCT02724618 Phase II | To determine the role of curcumin as a radioprotector against radiation-induced injury in normal tissues as well as a radiosensitizer in tumor in prostate cancer patients undergoing radiotherapy. | Recruiting participants |
| **Curcumin capsules** | dosage not provided | NCT00852332 phase II | To study how well giving docetaxel together with a curcumin works compared with giving docetaxel alone as first- or second-line therapy in treating patients with breast cancer. | Recruiting participants |
| **Isoquercetin** | 2 x 225 or 2 x: 450 mg/day along the chemotherapy with Sunitinib | NCT02446795 Phase I/II | A trial of isoquercetin as an adjunct therapy in patients with kidney cancer receiving first-line Sunitinib. | This study is not yet open for participant recruitment. |
| **Quercetin**   | 2 x 250 mg / day for 3 weeks | NCT01732393 | To evaluate the effect of quercetin on prevention and treatment of chemotherapy-induced oral mucositis in patients with blood malignancies. | This study has been completed, no results published |
| **SRT501** (micronized resveratrol) | 5 g/day for 14 days | NCT00920803 Phase I | To determine safety and tolerability of SRT501 in subjects with colorectal cancer and hepatic metastases | SRT501 was well tolerated. Mean plasma resveratrol levels following a single dose of SRT501 administration were exceeding those for equivalent doses of non-micronized resveratrol by 3.6-fold. Resveratrol was detectable in hepatic tissue. Cleaved caspase-3 was significantly increased [184]. |
| **PEITC** (dosage not provided) | NCT00691132 Phase II | PEITC in preventing lung cancer in people who smoke | The recruitment status unknown |

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**Phenylethyl isothiocyanate (PEITC), naturally occurring in cruciferous vegetables, has been widely studied**
for its biological activity and provedoxert anti-cancer properties. PEITC strongly induced oxidative damage due to depletion of glutathione and inhibition of GPX in H-Ras-transformed ovarian epithelial cells [98]. Depletion of cellular glutathione after PEITC treatment was observed in cancer cells of different origin, including glioma, oral cavity cancer, leukemia, prostate and breast [99-103]. Recent data demonstrate that PEITC caused inhibition of GST in glioma GBM 8401 cells, leading to massive ROS induction and causing cell death [104]. PEITC sensitized cancer cells so cisplatin in biliary by activating PEITC-induced depletion of overall GSH, which facilitated McI-1 glutathionylation, promoted McI-1 degradation and sensitized cell so cisplatin [105]. This data indicate that combined antitumor therapy based on synergistic effect of GSH depletion and strong oxidative stress induction lead to an effective cancer cell killing.

THE HIOREDOXIN SYSTEM

Thioredoxin system includes thioredoxin (Trx), thioredoxin reductase (TrxR) and diotinamide adenine dinucleotide phosphate (NADPH) (Figure 1). Thioredoxins have a conserved dithiol Cys-Gly-Pro-Cys motif in their catalytic site and participate in the reduction of oxidized proteins. Thioredoxin reductases balance the amount of reduced Trx by transferring electrons from NADPH oxidized catalytic sites. Humans express three thioredoxin reductase isozymes: TrxR1 (cytosolic), TrxR2 (mitochondrial), TrxR3 (testis-specific).

Thioredoxin reductase activity of thioredoxins is dependent on electron carriers for catalytic cycles of enzymes and protect proteins from aggregation or inactivation resulting from their oxidation [106]. Thioredoxins were described as regulators of a number of transcription factors like NF-kB, HIF-1α, VEGF, modulate matrix metalloproteinase-9 (MMP-9), here for promoting proliferation, angiogenesis and metastasis. Apart from maintaining cell redox balance, Trx1 can inhibit apoptosis by binding and blocking the activity of Apoptosis Signal-Regulating Kinase 1 (ASK1), decreasing cell adhesion so anti-cancer drugs [107-110]. Both Trx1 and TrxR1 are highly expressed in malignant cells, maintaining cell viability and protecting from apoptosis [111]. Blocking the activity of thioredoxin system lowers the cell’s detoxifying potential and ameliorates oxidative insult. Many compounds have been studied for their activity in modulating thioredoxin system in tumor cells.

NATURAL PRODUCTS TARGETING HIOREDOXIN SYSTEM

A study testing catechins for their potential inhibit TrxR1 found that a polyphenol abundant in dried leaves of white, green and black (EGCG), abrogated TrxR1 activity by directly targeting TrxR1iol groups. EGCG led to a significant decrease in HeLa cells viability [112]. EGCG anti-cancer effect was also studied in combination with lutein in head and neck cancer cell lines and in xenograft models, whereby synergistically promoted p53 activation and apoptosis induction, leading to growth inhibition and abrogation of tumor volume [113]. 3-hydroxyl containing flavonoids quercetin and myricetin suppressed growth of A549 cells due to inhibition of cellular thioredoxins. The observation correlated with the elevated oxidized thioredoxin levels and the reduced TrxR activity [114]. Pleurotin, an irreversible TrxR inhibitor displayed anti-cancer properties in MCF-7 breast cancer and HT-29 colon cancer cell lines. Inhibition of TrxR by pleurotin correlated with decreased protein levels of VEGF, HIF-1α and HIF-1α target genes in studied cell lines and in MCF-7 mouse xenografts [115]. EM23, a natural sesquiterpene lactone isolated from Elephantopus mollis was found to attenuate TrxR activity in CaSkI and SiHa cells by direct binding to its selenocysteine site. EM23-mediated inhibition of TrxR was followed by induction of ROS and apoptosis [116]. Chaetocin, a competitive substrate and inhibitor of TrxR, induced apoptosis in HeLa and glioma cells due to ROS induction [117, 118]. Curcumin, a polyphenol derived from Curcuma longa inhibited TrxR activity, leading to ROS generation in HeLa cells [107]. Javvadit al. (2010) exploited the potential of curcumin in radiosensitization of squamous carcinoma cells. Thankohe ability of curcumin covalently bind to the C-terminal region of TrxR1, curcumin strongly inhibited its function, enhanced free radicals burst and sensitized cells to radiotherapy [119]. Clinically used inhibitor of thioredoxin reductase, auranoxin, displayed synergistic lethality with GSH inhibitor piperlongumine in gastric cancer (GC) suggesting that combined inhibition of different antioxidant systems is more effective in killing cancer cells than abrogation of activity of single one. t again expressed as a ROS scavenger as potent anticancer drug targets [120].

SUPEROXIDE DISMUTASE

Superoxide dismutase (SOD) drives the reaction of dismutation of superoxide into hydrogen peroxide (Figure 1). There are three types of SOD in cells: CuZnSOD (SOD1) abundant in cytosol, mitochondrial manganese superoxide dismutase MnsOD (SOD2) and extracellular ECSOD (SOD3). All superoxide dismutases carry metal ions in their active sites: SOD1 and SOD3 have zinc and copper and SOD2 carries manganese. SOD1 is mainly localized in the cytosol, but it has also been found in the outer mitochondrial membrane, where it neutralizes O₂⁻ released from Complex II. SOD2 is localized in the mitochondrial while SOD3 remains in the extracellular matrix and prevents oxidative stress damage [121]. MnSOD overexpression...
is common in tumors and contributes to therapy resistance. SOD neutralizes toxic superoxide, but as a consequence creates hydrogen peroxide, which can be further neutralized by catalase and glutathione cycle [122].

NATURAL PRODUCTS BLOCKING SOD ACTIVITY

Since mitochondria are the primary source of cellular free radicals, decreasing their detoxifying ability by means of blocking SOD2 activity inms might contribute to apoptosis activation. Plumbagin proved sufficiently induce apoptosis in prostate cancer cell lines, partially through decreasing SOD2 expression [97]. PEITC was found to inhibit expression of SOD in LN229 glioma cell line, weakening cellular antioxidant defense and causing apoptosis [99]. Suppression of SOD enzymatic activity by apigenin in combination with ROS-inducing paclitaxel was found to sensitize HeLa cells to apoptosis and allowed lower paclitaxel doses [123].

CATALASE (CAT)

Catalase is a peroxisomal enzyme that neutralizes hydrogen peroxide by its decomposition to water and oxygen (Figure 1). High levels of hydrogen peroxide facilitate DNA mutagenesis herefore under physiological conditions catalase protects cells from oxidative damage. H2O2 also serves as mediator of apoptosis and can modify regulatory protein complexes, such as Nrf2/Keap1 system. Apart from peroxisomal CAT, malignant cells acquire membrane-associated catalase to survive under oxidative stress [124-126]. Blocking catalase activity can significantly increase oxidative burden through hydrogen peroxide accumulation which triggers tumor cells death.

NATURAL PRODUCTS INHIBITING CATALASE

Wogonin, a flavonoid isolated from Scutellaria baicalensis was shown to induce cell death in cervix, ovary and lung cancer cell lines through catalase inhibition that increased hydrogen peroxide levels and facilitated TNF-induced apoptotic signaling [127]. Human hepatoma HepG2 cells subjected to apigenin accumulated H2O2, which correlated with a decrease of catalase mRNA and catalase activity and led to cell death [128]. PEITC treatment lowered catalase protein levels and induced ROS in GBM 8401 glioma cells [104]. EGCG inhibited catalase activity both in vitro and in K562 cells [129] and sensitized cells to arsenite (As) treatment. The proposed mechanism explained the inhibition of catalase activity upon treatment with As/EGCG occurred via JNK (c-Jun N-terminal kinase) signaling pathway. Genotoxic stress that activated JNK, promoted catalase phosphorylation by c-Abl kinase, marking it for proteasomal degradation. Blocking catalase activity led to high amount of H2O2 and promoted death of epithelial cells subjected to As/EGCG [130].

EXOGENOUS ANTIOXIDANTS

The role of oxidative stress in initiating and promoting cancer on one hand and in causing oxidative damage on the other justifies the opposite ROS-manipulating strategies against cancer. First is antioxidant approach functional in cancer prevention and therapy. The most important and widespread exogenous dietary antioxidants are vitamins A and E, their analogs carotenoids and tocopherols, vitamin C and polyphenols. Though preventing ROS-induced mutations and subsequent cancer initiation with dietary antioxidants is well documented, their use during anticancer therapy remains controversial. Since cancer therapy highly relies on the production of free radicals, it has been speculated that supplying cells in antioxidants might decrease treatment efficacy. On the other hand, the basic idea behind using antioxidants during therapy isoliminates excessive oxidative damage and to help alleviate adverse effects. Many patients undergoing therapy areaking antioxidants without consulting with a physician. Selenium and vitamin C are widely used in complementary oncology [131]. Radiotherapies in head and neck cancers showed that vitamin E Euedehoxidicity, however overall recurrence and mortality were assessed [132, 133]. Trials on the effect of antioxidants on chemotherapy reported on some benefits of using vitamin E or selenium with cisplatin, axol and oxiplatin, but the long-term effects were not assessed [134-138]. Decreased recurrence of some cancers in patients otceiving treatment or after chemotherapy has also been reported [139, 140]. The main conclusion from these trials is that administration of antioxidants to cancer patients in combination with therapy should be taken with great care. Patient phenotype (smoking, alcohol uptake and nutrition), tumor localization (different partial pressures of oxygen among tissues) and type of therapy should be considered in order to choose a suitable antioxidant supplement [141]. Importantly, adverse effects were reported with antioxidants derived from food. The Women’s Healthy Eating and Living Study (WHELS), where diet consisted of high amount of fruit and vegetable, showed that supplementation with vitamin C, showed to be effective in patients with early breast cancer [142].

LESSONS FROM CLINICAL TRIALS

Anticancer properties of a few natural products from Table 1 (EGCG, curcumin, everolimus, PEITC, have been studied clinically mainly in the context of decreasing side effects caused by chemotherapy and adjuvant therapy or as chemopreventive dietary supplements (Table 2).
The serials were basing on ROS scavenging properties of natural compounds. The ability of orally administered EGCG to reduce incidence and severity of otosphenitis was tested in patients with locally advanced stage T1N0M0 small-cell lung cancer receiving concurrent chemotherapy and adjuvant radiotherapy (phase, NCT01481818). No dose-limiting toxicity of EGCG was reported. Dramatic regression of otosphenitis grade 0/1 was observed in 22 of 24 patients and the pain score was also reduced [143]. Currently, the EGCG-mediated protection of the esophagus from damage induced by radiotherapy in patients with lung cancer is being tested in phase I (NCT02577393). Also, orally administered EGCG was shown to be effective in decreasing radiation dermatitis in patients with breast cancer after mastectomy or receiving adjuvant radiotherapy [144]. Orally administered curcumin significantly reduced the severity of skin reactions (dermatitis) caused by adjuvant therapy breast cancer patients as shown in phase II clinical trials (NCT01246973) [145] and prevented colon cancer by reducing the aberrant crypt foci (ACF) number in smokers at dose 4 g/day [146]. Unfortunately, just a few trials so far addressed a question on whether natural compounds could improve the efficacy of chemotherapy or radiation therapy. One such trial (phase I) tested curcumin ability to potentiate the effect of gemcitabine in patients with advanced pancreatic cancer (NCT00192842). n out of twenty patients showed a substantial increase in plasma curcumin levels after oral administration with a maximum concentration of 25 µM. This suggests that curcumin may have potential as an adjuvant for gemcitabine therapy.

CONCLUSIONS

The power of natural products lies in using them as adjuvants to standard anticancer therapies. The struggle is that in some cases, they may exhibit contrary actions, depending on concentration. At high doses ( > 50 µM), natural compounds cannot be expected to have any oxidant properties due to their antioxidant capacity, which may limit their effectiveness in cancer treatment.

Abbreviations

ABCC2, ABC transporters MRP2; Akt, Protein Kinase B; AP-1, activator protein 1; ARE, antioxidant response element; As, arsenite; ASK1, apoptosis signal-regulating kinase 1; BCRP, breast cancer resistance protein; B-Raf(V619E), mutation in B-Raf that places a valine with glutamic acid at position 619; c-Abl, Abelson tyrosine kinase; CAT, catalase; COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; Cul,
cullin; EGCG, pigallocatechin gallate; EMT, pidermal mesenchymal transition; EGFR, Epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; ETC, electron transport chain; GC, gastric cancer; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; GST1α/β, Glutathione S-transferase 1α/β; H₂O₂, hydrogen peroxide; HIF-1α, Hypoxia-inducible Factor 1α; HNSCC, head and neck squamous cell carcinoma; H-Ras(G12V), mutation in H-Ras that places amino acid glycine with valine at position 12; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; K-Ras(G12D), mutation in K-Ras that places amino acid glycine with aspartic acid at position 12; K-Ras, V-Ki-ras2 Kirsten sarcoma viral oncogene homolog; Mcl-1, induced myeloid leukemia cell differentiation protein; MEFs, murine embryonic fibroblasts; MMP-9, matrix metalloproteinase-9; mtp53, mutant p53; Myc, myelocytomatosis oncogene; Myr-Akt, myristoylated form of Akt kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NCI, National Cancer Institute; NfκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOX, NADPH oxidases; NOX1, NADPH oxidase 1; NOX5-L, NADPH oxidase 5; NQO1, NAD(P)H quinone oxidoreductase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; O₂•−, superoxide anion; OH•, hydroxyl radical; OONO−, peroxynitrite; p38, MAPK14, Mitogen Activated Protein Kinase 14; p47phox, phagocyte NADPH oxidase/NOX2 organizer; PDK1, 3-Phosphoinositide Dependent Protein Kinase 1; PI3K, phosphoinositide 3-kinase; PKCδ, Protein Kinase 1; PEITC, phenylethyl isothiocyanate; PL, piperlongumine; ROS, reactive oxygen species; SH, sulfhydryl group; SOD, superoxide dismutase; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; SOD3, superoxide dismutase 3; STAT3, signal transducer and activator of transcription 3; TRAIL, TNF-related apoptosis-inducing ligand; Trx, thioredoxin; Trx1, thioredoxin 1; TrxR, thioredoxin reductase; TrxR1, thioredoxin reductase 1; UGT, UDP-glucuronosyltransferases; VEGF, Vascular Endothelial Growth Factor; V-Ras, Neuroblastoma RAS viral (V-Ras) oncogene homolog; WHELS, Women’s Healthy Eating and Living Study; γGCS, γ-glutamylcysteine synthetase.

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CONFLICTS OF INTEREST

Authors declare no conflict of interest regarding this article.

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