Review

Role of Reactive Oxygen Species and Targeted Therapy in Metastatic Melanoma

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Abstract
Reactive oxygen species (ROS) play a significant role in various stages of melanoma development including melanocyte transformation, melanin production, melanoma cell metabolism, metastasis and immune response against melanoma progression. Several molecular and enzymatic signaling cascades control ROS levels and have differential regulation depending on the cell type. The equilibrium between ROS production and scavenging is crucial for maintaining cellular homeostasis and this balance is often altered in tumor cells. ROS is generated in cancer cells due to mitochondrial dysfunction, enhanced metabolic rates, increased cellular signaling, enhanced peroxisome activities and genetic alterations. In this review, we discuss the source and mechanisms of ROS generation. We also highlight the role of ROS in the process of melanomagenesis. This review provides an overview of ROS-dependent anticancer therapies including ROS scavenging antioxidants and ROS boosting therapies which have presented promising outcomes both in *in vitro* and *in vivo* melanoma models. We summarize how the understanding of ROS-targeted signaling plays a crucial role in melanoma prognosis and drug resistance. Hence, the knowledge of ROS in melanoma etiology and progression can be exploited in clinical practice for development of better therapies for melanoma treatment.
Keywords: Melanoma, ROS, BRAF, antioxidant, targeted therapy

Introduction

Melanoma is a highly malignant form of skin cancer derived from melanin producing melanocytes which protect the skin from UV-induced radiation damage. Although melanoma cases represent only 4% of all skin cancers, it accounts for 80% of all skin cancer related deaths. Melanoma five year survival rates are at only 15% for patients with advanced disease, which is incurable, underscoring the urgent need for more optimal therapies. According to the American Cancer Society, about 91,270 new cases of melanoma will be diagnosed in the US in the year 2018, and 9,320 people are expected to die from melanoma in 2018 (1). The etiology of melanoma is poorly understood, however various molecular and cellular mechanisms are known to contribute towards melanoma development. Melanoma metastasis is characterized by several intermediate processes including angiogenesis, extravasation, evasion of immune surveillance, presence of prothrombotic embolism, adhesion and organ specific colonization (2). Melanoma metastases can develop in regional lymph nodes as in-transit

lesions or a satellite or in distant organs. Lymph flow and chemotaxis regulate the homing of melanoma cells to other distant sites. Molecular markers such as p53, BRAF, N-RAS, cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase inhibitor 2A (CDKN2A), c-KIT, melanocortin 1 receptor (MC1R), mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1) and cadherins contribute to melanoma metastasis (2, 3). Several signaling pathways including mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and phosphoinositide 3-kinase (PI3K) cascades induce the development of melanoma through various genomic alterations. Approximately 50% of melanomas contain a $BRAF^{T1799A}$ transversion, encoding the constitutively active $BRAF^{V600E}$ oncoprotein. This led to the clinical development of selective adenosine triphosphate (ATP)-competitive RAF kinase inhibitors, including vemurafenib and dabrafenib, targeting the mutant BRAF protein (4, 5). Combination therapy including BRAF and MEK inhibitors has become the standard of care for BRAF-mutant melanoma with response rates of ~70% (6, 7). However, most of the patients acquire resistance and eventually exhibit relapse and disease progression. There is evidence suggesting that increased ROS in tumor cells leads to oxidative stress and initiate signaling pathways leading to persistent tumor cell survival, vascularization and metastasis which ultimately results in the resistance to clinically relevant drugs (8).

ROS is generated due to enhanced metabolism of transformed cancer cells, immune reaction against developing tumors and DNA damage induced due to ultraviolet radiations. There are several incongruities regarding the role of ROS in regulating cell growth and the mechanism leading to its generation. ROS acts as a double edge sword which exhibits beneficial or deleterious effects depending on cell type, levels and types of ROS involved or genetic background of the living systems. Low/moderate ROS levels are essential for normal cell survival and proliferation; however increased ROS production can lead to oxidative stress which in turn damages cellular proteins, DNA and lipid components. In cancerous cells, enhanced ROS production can lead to activation of signaling cascades and metabolic activities that promote ROS adaptation, ultimately leading to upregulation of antioxidants to maintain the redox homeostasis (9, 10). The balance between benign and deleterious effects of ROS is controlled by redox regulation and is a decisive factor for survival of both normal and cancer cells. Elevated production of ROS and an altered redox status has long been observed in cancer cells including melanoma. High ROS triggers the metastatic potential of melanoma via inducing DNA changes (mutations and epigenetic alterations), activating cell proliferation, stimulating the adhesion of circulating tumor cells (CTCs) to the blood vessels to promote extravasation, escaping immune surveillance, damaging the microenvironment that secrete chemokines to destroy the melanoma cells. It also induces the activation of pro-metastatic molecules such as urokinase plasminogen activator receptor (uPAR), C-X-C chemokine receptor type 4 (CXCR4), interleukin-8 (IL-8), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and very late antigen 4 (VLA-4) expression which promote melanoma metastasis (11). Migratory bone marrow-derived cells (BMDCs) and tumor-associated macrophages fuse with melanoma cells and contribute to melanoma metastasis via signaling molecules which regulate epithelial-mesenchymal transition (EMT) pathways (12). Recent observations demonstrate that BRAF inhibitors induce ROS generation in melanoma cells through peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α)-induced mitochondria biogenesis (13). Furthermore irrespective of PGC1α status, ROS production is induced by BRAF inhibition (14). Hence, antioxidants or therapies that utilize elevated ROS levels can be used as monotherapy or as combination therapy with standard of care treatments may serve as a promising alternative for patients with malignant melanoma.

Transformation of melanocytes to malignant melanoma

Malignant melanoma is a neoplasm which arises from malignant transformation of melanocytes present at the basal epidermis of skin but also found at other sites including mucosal membrane (maxillary gingiva, hard palate, lip, esophagus, throat, vulva, perianal region) and eye (uvea and retina). Melanoma is highly heterogeneous in outcome. All the histological and

clinical patterns of melanoma are primarily caused by UV irradiation, with the incidence being markedly augmented in patients with chronic sun exposure and repeated episodes of sunburn (15). Several signaling cascades are differently involved between types and subtypes of melanoma classified according to anatomic site or sun exposure (16). Consequently, a mutation in BRAF and N-RAS commonly prevails in melanoma that is found at sites intermittently exposed to UV, while KIT mutations are found at chronically sun-exposed or sun-protected sites like mucous membranes (17, 18). Ultraviolet (UV) radiations can induce DNA damage through direct and indirect mechanisms. These radiations are subdivided into longer wavelength UVA, shorter UVB and UVC. In addition, UV radiation locally induces several cytokines, proopiomelanocortin peptides, enkephalins, urocortins, corticotropin producing hormones and release them into local circulation to exert systemic effects including activation of the central hypothalamic-pituitary-adrenal axis, opioidogenic effects and immunosuppression (19). UVC radiations possess minimal harmful effects as these are readily absorbed by ozone layer in the atmosphere. UVB radiations are absorbed by the outer epidermis, cause DNA breaks, carcinogenesis in the keratinocytes via formation of mutagenic cyclobutane pyrimidine dimers and photoproducts. This could lead to development of non-melanoma skin cancer (20). Also, UVB radiations cause indirect DNA damage via oxidative stress generation (21). UVA radiations are highly penetrating which are readily absorbed by keratinocytes, melanocytes and dermal fibroblasts (22). UVA induced melanocytes transformation is through two different mechanisms, depending on the different precursor lesions which include different gene mutations and stages of transformation as discussed (23). Melanomas associated with chronic sun exposure due to UVA radiation don't arise from preexisting nevi, but from melanoma *in situ* or dysplastic lesions containing several mutations in proto-oncogene and tumor suppressor gene including tumor protein p53 (TP53), neurofibromatosis type I (NF1), and phosphatase and tensin homolog (PTEN). However, benign nevi harboring 80% of $BRAF^{V600E}$ mutation remain indolent for decades due to immune surveillance. These nevi require activation of additional mutations in telomerase reverse transcriptase (TERT) and CDKN2A for their malignant transformation (23-25). UVA and UVB radiations both contribute to light induced carcinogenesis and immune suppression (26). A recent study demonstrates that malignant transformation of melanocytes in melanoma cell lines and patient tumors follows a two-dimensional differentiation trajectory which is attributed to the embryonic history of melanocytes derived from four subtype clusters. The first cluster is the undifferentiated subtype due to enrichment of invasive phenotype genes including those involved in cell migration and adhesion. The second is defined as neural crest-like subtype because of unique enrichments for neural crest-related gene sets. The third subtype is transitory subtype due to mixed enrichment of pigmentation-associated genes and neural crest-associated gene types. The fourth is the most differentiated melanocytic subtype due to loss of neural crest signature and enrichment of pigment-associated gene sets. This differentiation model can be linked with subtype-specific sensitivity to iron-dependent oxidative stress and non-apoptotic cell death termed as ferroptosis. In addition, this differentiation pattern plays a key role in recurrent innate/acquired resistance mechanisms to kinase targeted therapies and immunotherapies in the clinic (27). Figure 1 illustrates the transformation of normal melanocytes to malignant melanoma and the associated signaling mechanism.

Melanin synthesis and its hormonal regulation

Melanin represents a group of natural pigments classified as eumelanin, pheomelanin, neuromelanin which are synthesized inside melanocytes as the end products during multistep transformation of L-tyrosine. Melanin biosynthesis is initiated either directly from L-tyrosine or the hydroxylation of L-phenylalanine to L-tyrosine that is further hydroxylated to L-dihydroxyphenylalanine (L-DOPA). L-DOPA is oxidized to dopaquinone, a common intermediate to eu- and pheomelanogenic pathways (28). Dopaquinone is transformed into leukodopachrome during the process of eumelanogenesis followed by several oxido-reduction reactions with production of the intermediates dihydroxyindole (DHI) and DHI carboxylic acid (DHICA) along with the final synthesis of eumelanin. Dopaquinone conjugates with cysteine or glutathione

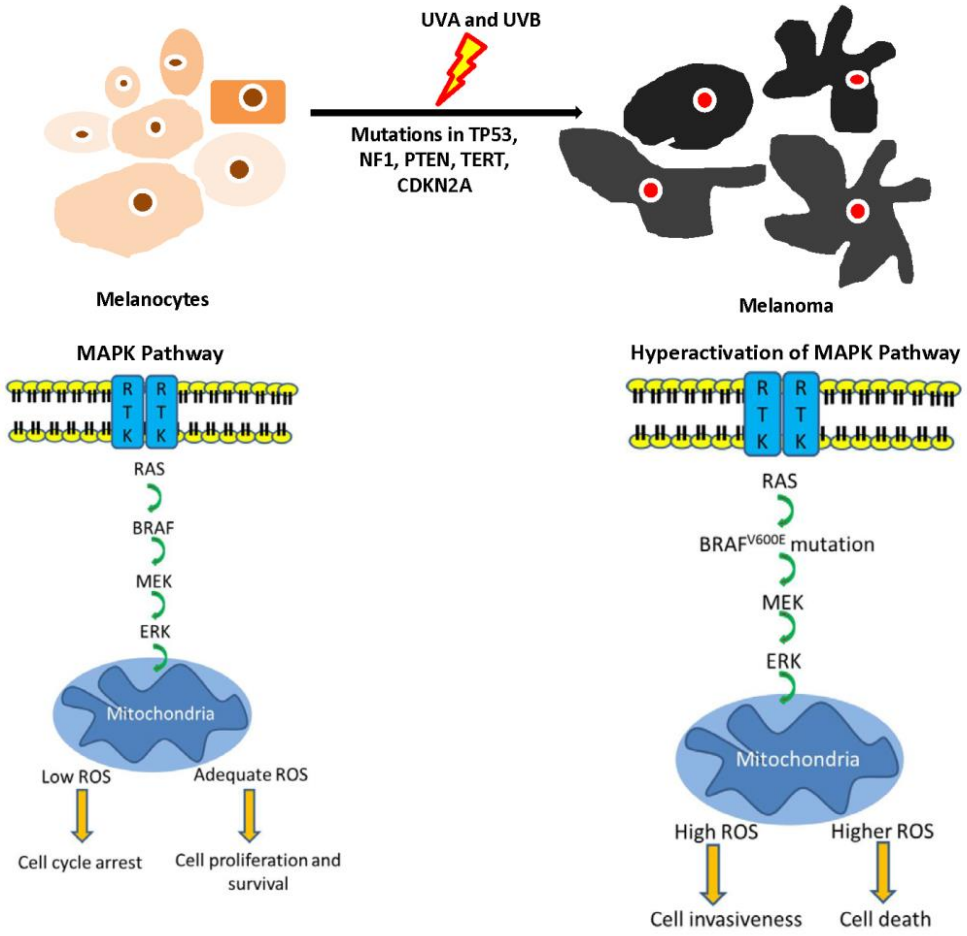

Figure 1. Transformation of normal melanocytes to malignant melanoma and associated signaling mechanism. Factors contributing to malignant transformation of normal melanocytes. It depicts normal and hyperactivated MAPK pathway and consequences of altered ROS levels in normal melanocytes versus malignant melanoma cells.

(GSH) to yield cysteinyldopa and glutathionyldopa along with the final production of pheomelanin during the process of pheomelanogenesis. The types of intermediates and rate of reaction are tightly regulated by physicochemical milieu including pH, metal ions and tyrosinase related protein type 1 (TRP1) and tyrosinase related protein type 2 (TRP2) (28, 29). L-tyrosine and L-DOPA not only serve as substrate and intermediate in the process of melanogenesis but also act as positive regulators and inducers in the melanogenic pathway through receptor or non-receptor-mediated processes (30). Based on L-DOPA and L-tyrosine concentrations, membrane-associated binding proteins lead to formation of melanosomes and delivery of elements necessary for melanogenesis through endocytic signaling pathways (31). There are several hormones including melanocortins (MSH) and adrenocorticotropic hormone (ACTH) and their receptors which regulate the process of melanogenesis. Several other growth factors and neurotransmitters also modulate various steps involved in melanogenesis (32). The systemic administration of ACTH, α-MSH and ß-MSH induce pigmentation in sun-exposed areas of human skin (33, 34). In melanoma cells, α-MSH and ß-MSH trigger

melanogenesis by activating tyrosinase and post-dopa oxidase steps (35). In cultured melanocytes derived from human skin, minimal concentration of α-MSH, ß-MSH and ACTH stimulate melanocyte proliferation, cyclic adenosine monophosphate (cAMP) synthesis and tyrosinase activity via interaction with MC1R (36). ß-endorphin (a secretory hormone) and its μ-opiate receptor are widely expressed in human epidermal melanocytes and closely associated with melanocyte proliferation, dendricity and pigmentation. ß-endorphin/μ-opiate receptor complex signals via protein kinase C (PKC) ß-isoforms or at gene transcriptional level to upregulate tyrosinase activity with stimulation of melanogenesis (37). Endothelin, a vasoconstrictive peptide synthesized by endothelial cells plays a key role in melanocyte proliferation and differentiation (38). Cultured human melanocytes stimulated by endothelin results in increased tyrosinase activity via activation of PKC, cAMP and PKA signaling pathways (39). Histamine, a neurotransmitter known to be involved in inflammatory response regulates the ratio of eumelanin-to-pheomelanin in cultured human melanocytes (40). Different histamine receptor agonists differentially regulate tyrosinase activity and the process of melanogenesis (41). The catecholamine agonists including those against epinephrine, norepinephrine modulate tyrosinase activity and melanin synthesis (42). Stem cell factor (SCF) also known as KIT ligand or mast cell growth factor is encoded by c-kit proto-oncogene which regulates melanocyte differentiation and melanin pigmentation (43, 44). Several steroid hormones and their receptors modulate melanogenesis by regulating tyrosinase activity. Estrogen effect on tyrosinase activity is influenced by multiple factors including cultured conditions of human melanocytes (45, 46). Vitamin D and its derivatives differentially regulate melanocyte proliferation, tyrosinase activity or melanogenesis. A study found that vitamin D3 induces the tyrosinase activity (47) and another report suggests no significant effect on its activity (48). Another study indicates that human melanocytes stimulated with vitamin D3 do not affect melanin synthesis, melanocytes proliferation or tyrosinase activity whereas vitamin D3 derivatives including $25(OH)D_3$ and $1,25(OH)_2D_3$ suppress tyrosinase activity via stimulation of endothelin B receptor expression (49). Melanogenesis is negatively regulated by several G- protein coupled ligands (serotonin, melatonin, dopamine and acetycholine) and their receptors, melanocortin antagonists (agouti proteins, melanin concentrating hormones), cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and their receptors. Other potent inhibitors of melanogenesis are ceramide-2, triiodothyronine (T3) and vitamin E (32).

Melanogenesis in regulating melanoma behavior and therapy

Melanogenesis is a tightly regulated process leading to melanin synthesis which protects the epidermis and normal melanocytes against UV-induced damage via acting as radioprotector, light filter and scavenger of free radicals, metal cations and toxic chemicals. These properties although exhibit beneficial effects on normal skin however make the melanotic melanomas resistant to chemo-, radio- and phototherapy (32). It also affects epidermal homeostasis and melanoma behavior via modulating various signaling pathways (50). Induction of melanin pigmentation in cultured melanoma cells results in robust upregulation of HIF-1-dependent target gene expression involved in the process of angiogenesis (vascular endothelial growth factor A; VEGFA), cellular metabolism including glucose metabolism (glucose transporter 1; GLUT1) and also stimulates key enzymes in the glycolytic pathway including pyruvate dehydrogenase kinase 1 (PDK1), hexokinase 2 (HK2), aldolase A (ALDOA), lactose dehydrogenase A (LDHA). In addition, melanogenesis stimulates several stress, steroidogenic, immune and growth-related genes having putative hypoxia response elements (HREs) binding sites indicating the role of melanogenesis in HIF-1-independent pathways. High concentration of melanogenic precursors in culture media of hamster AbC1 and SK-MEL-188 melanoma cells leads to overall increase in HIF-1α and but not HIF-2α expression. Nuclear HIF-1α expression is significantly higher in excisions of advanced melanotic compared to amelanotic melanomas (51). Melanin synthesis is linked to higher disease advancement in patients with metastatic melanoma and reduces the outcome of radiotherapy (52). It also shortens overall and disease-free survival of patients with metastatic melanoma (53). The process of melanogenesis acts as a double

edge sword where it generates melanin to scavenge ROS and other toxic agents on one hand; while on the other induces ROS, quinone, semiquinone intermediates production which display genotoxic, cytotoxic or mutagenic activities and act as potent immunosuppressant. The intermediates produced in the melanogenesis process exhibit immunosuppressive effects by shutting off T- and B-cell immune activities or the selective lymphotoxic effects of levodopa or products of its autoxidation (30, 32, 54-56).

Reactive Oxygen Species and its source

ROS are a group of chemically reactive molecules comprising of a family of radical and non-radical species derived from partial reduction of molecular oxygen. A radical species is a free electron-containing species that includes superoxide anion ($O_2•^-$), its conjugated hydroperoxyl radical ($HO_2•^-$), hydroxyl (•OH), carbonate ($CO_3•^-$), peroxyl ($RO_2•$) and the alkoxyl radical (RO•). Non-radical species like hydrogen chloride (HCl), hydrogen peroxide (H_2O_2), reactive aldehydes, fatty acid hydroperoxides (FaOOH), reactive aldehydes and singlet oxygen which can be readily reduced into free electron-containing species (57). The way ROS reacts with different compounds varies, based on the processing into higher reactive species and their diffusion capabilities. Certain ROS including H_2O_2 and $O_2•^-$ readily diffuse away from the site of formation into extracellular space and are more stable as these molecules don't interact with other biomolecules. Hydroxide free radicals on the other hand are highly reactive with a very short half-life. •OH radicals produced from the reaction of iron and hydrogen peroxide are very reactive as they readily react with species in the immediate environment *in vivo* (58). The ROS generation traditionally is attributed as a byproduct of cellular metabolism during mitochondrial electron transport chain (ETC) (59). Other cellular compartments including peroxisomes, melanosomes, and enzymes including NADPH oxidases (NOX) family members are also known to contribute to ROS generation (60). Isolated mitochondria are known to produce $O2•^-$ through autoxidation of the flavin component of complex I nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) and/or autooxidation of the semiquinone at complex III. Approximately 1-5% of total oxygen consumed in aerobic metabolism leads to formation of $O2•^-$ which is produced by complex I and is released into mitochondrial matrix via electron leaks. Complex III releases $O_2•^-$ both into inner mitochondrial space and matrix (61, 62). Mitochondrial DNA mutation is not the major cause for ROS synthesis and cancer development in melanoma. However, the mitochondria plays a key role in defective metabolic regulation and mitochondrial-derived ROS directly participate in the process (63). In addition, some reports suggest the role of mitochondrial-derived ROS in cancer metastasis (64, 65).

Another source of ROS is NADPH oxidases, a NOX family member which contain membrane-bound enzymes catalyzing the controlled production of $O_2•^-$ via coupling NADPH-derived electrons to oxygen. NADPH oxidase complex contains cytochrome B559 with two subunits of gp91phox, p22phox and four cytoplasmic proteins, p47phox, p67phox, p40phox and the small guanosine triphosphate (GTP) binding Ras-related C3 botulinum toxin substrate 1 (RAC1) and RAC2 (66, 67). The NOX family of enzymes includes 7 family members (Nox1-5, Duox1 and Duox2) and acts as a major contributor of cytosolic ROS. Several pieces of evidence suggest the key role of cytosolic ROS in melanoma progression. Nox1 overexpression in Wm3211 primary melanoma cells induces ROS production, increases cell invasion via upregulation of matrix metallopeptidase 2 (MMP-2) protein expressions and regulation of the EMT pathway (68). Hyaluronic acid (HA) stimulates Rac1 activity, induces Nox1 activity which enhances ROS generation and B16F10 cell motility (69). H_2O_2 induces ROS production via Rac1/Nox1-dependent mechanism which in turn induces the pro-metastatic property of mouse non-invasive B16F0 melanoma cells (70). Nox4 expression is associated with melanoma development and Nox4-generated ROS contributes to melanoma growth via regulating the G2-M cell cycle arrest (71). Superoxide anions are also generated through electron transfer reaction catalyzed by enzymes including xanthine oxidases, cyclooxygenases, monoamine oxidases, lipoxygenases and components of the cytochrome P450 system. Peroxisomes under physiological conditions generate H_2O_2, but not $O2•^-$ (72). Several studies indicate that melanocytes and melanoma cells

demonstrate a unique redox regulation that led to discovery of ROS generating roles of melanin and melanosomes in melanoma progression (73-75).

Role of ROS in initiation and melanoma progression

ROS is involved in several stages of melanomagenesis which includes ROS-induced malignant transformation of hypoxic melanocytes, melanoma cell metabolism, immune response towards melanoma, melanin synthesis and melanoma metastasis. Hypoxia inducible factor-1 alpha (HIF-1α) activation is necessary for AKT-dependent transformation of melanocytes residing in the mildly hypoxic environment of skin epidermis (76). AKT induces the expression of the ROS generating enzyme Nox4 in melanoma cells and also stabilizes cells that generate ROS due to severe mitochondrial damage (77). MAPK signaling is utilized by melanoma cells to regulate ROS synthesis which in turn maintains melanoma cell survival by modulating apoptotic signaling pathways (78). ROS constitutively activates the (nuclear factor kappa-light-chain-enhancer of activated B cells) NF-κB pathway to promote melanoma progression (79). ROS activates another transcription factor, activator protein-1 (AP-1) which plays a crucial role in Ras-induced oncogenic transformation (80, 81). It is reported that ROS-induced cell death of melanoma cells contributes to vasculogenic mimicry, the formation of microvascular channels by tumor cells (82). Metabolically active melanoma cells can impair ROS homeostasis by modulating the activity of growth promoting signaling pathways. ROS generated by NADPH oxidase increases melanoma cell proliferation via NF-κB pathway activation (83). Inflammatory immune cells including mast cells, monocytes/macrophages infiltrate the microenvironment of melanoma during early and late stage of tumorigenesis. These cells are capable of producing ROS as a cytotoxic mediator to kill cancer cells. ROS generated by these inflammatory cells can induce permanent genomic alterations such as mutations, rearrangements and deletions leading to development of genetically different tumor cells which exhibit resistance to oxidative stress pressure (84). UV radiation-induced melanin synthesis is a complex phenomenon which results in formation of basic monomers for red/light brown phaeomelanin. Cysteine is a necessary amino acid involved in phaeomelanin production that plays an important part in defense mechanism against ROS signaling. More production of phaeomelanin leads to depletion of glutathione and causes oxidative stress. As a result phaeomelanin and 5-S-cysteinyldopa become pro-oxidants in melanoma cells compared to normal melanocytes (85). Binding of iron to melanin oxidizes melanin which reacts with O_2 to form H_2O_2, $O_2\bullet^-$ and other free radicals which perturb the ROS homeostasis (86). UVA induces thioredoxin interacting protein (Txnip) expression which consequently suppresses Trx (thioredoxin) activity. This process results in enhanced ROS and transendothelial cell migration of melanoma cells which promotes early stage melanoma metastasis. High ROS inhibits Txnip expression, thereby pushing cells to inhibit ROS levels (87). The *in vivo* metastasis is also dependent on interaction of ROS with the tumor microenvironment and activation of HIF-1α regulated genes (88, 89).

ROS and redox signaling in melanoma

Tumor cells exposed to high ROS levels activate several redox sensors in order to adapt to the oxidative stress for maintenance of homeostasis. Apurinic/apyrimidinic endonuclease 1 (APE-1)/Ref-1 activates several transcription factors such as AP-1, HIF-1α, NF-κB and p53 which modulate cell survival, proliferation, and apoptotic signaling pathways (90). Increased APE-1/Ref-1 is associated with decreased ROS and reduced DNA damage lesions induced due to oxidative stress. However, a prolonged oxidative stress leads to continuous activation of APE-1/Ref-1 that switches cellular signaling to sustained proliferation. Yang et al. shows that enhanced ROS and APE-1/Ref-1 contribute significantly to malignant transformation by increased soft agar colony formation and anchorage-independent growth (91). The same group also demonstrates that abnormal high levels of APE-1/Ref-1 in melanoma cells versus normal melanocytes can lead to melanoma cell proliferation and drug resistance (92). Ras-ERK pathway is activated by superoxide anion level which is associated with global DNA hypermethylation responsible for malignant transformation of melanocytes (93). Another MAPK signaling pathway, p38 is activated in melanocytes exposed to oxidative stress and UV-induced irradiation. Activated p38 in turn induces the tumor suppressor $p16^{INK4A}$ which in turn decreases ROS

levels (94). The p38 MAPK pathway is reported to play a significant role in ROS-induced apoptosis (95, 96).

Antioxidant therapy in melanoma

Redox homeostasis in normal or tumor cells including melanoma is determined by the balance between the ROS production and detoxification rates by various antioxidant defense systems. The body has a number of ways by which it can induce formation of antioxidants in response to increased oxidative stress. The maintenance of intracellular redox state is crucial for regulation of signal transduction pathways in both normal and cancer cells. Based on this, an option for addressing increased ROS levels would be to augment the levels of antioxidants in melanoma patients. Literature both supports and opposes the role of antioxidants in melanoma development. Antioxidant defense systems are broadly categorized into enzymatic and non-enzymatic sub-types. ***Enzymatic antioxidants defense system*** consists of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT). SODs convert the $O_2\bullet^-$ into the lesser reactive species H_2O_2 (97). There are three SODs involved in the superoxide neutralization process depending on the site of production and diffusion. MnSOD2 is present in the mitochondria, Cu,Zn-SOD1 is present in the cytoplasm, Cu,ZnSOD3 is located in the extracellular space (98). SOD inhibits the vemurafenib-induced upregulation of intracellular $O_2\bullet^-$ and nitric oxide (NO) production, thereby salvaging cell proliferation in human melanoma A375 cells harboring $BRAF^{V600E}$ mutation (99). Increased MnSOD2 expression alters the malignant phenotypes of melanoma cells in culture, reduces colony formation and tumor growth in nude mice model (100). Catalase decomposes H_2O_2 to H_2O and O_2 in peroxisomes. Polyethylene glycol (PEG)-conjugated catalase shows a beneficial effect in *in vitro* and *in vivo* melanoma models in which it lowers the expression of epidermal growth factor (EGF) and EGFR in the lungs of rodents harboring metastatic melanoma tumor cells. PEG-catalase inhibits the initial stages in metastasis by reducing the number of surviving tumor cells and is also significantly effective in later stages of melanoma progression (101). Taken together, PEG-catalase inhibits the survival, adhesion, invasion and proliferation both in *in vitro* and *in vivo* melanoma models by inducing tumor dormancy and hence prolonging tumor survival period (102). Glutathione catalyzes the reduction of H_2O_2 into H_2O via oxidation of glutathione. Glutathione is present in both reduced (GSH) and in oxidized (GSSG) forms. The ratio of GSH/GSSG tightly regulates redox homeostasis in the mammalian cells (103). High GSH levels protect metastatic murine melanoma B16 cells from *in vivo* and *in vitro* sinusoidal cell-mediated oxidative stress, thereby contributing to metastatic cell survival within the hepatic microvasculature (104). Peroxiredoxins (Prxs) are a family of peroxidases which reduce H_2O_2 into H_2O. Prxs isoforms are maintained in the reduced state by the thioredoxin reduction system in conjunction with GSH (105). The thioredoxin enzyme system consists of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH. Ectopic expression of Prx2 inhibits melanoma cell migration and proliferation via negatively regulating ERK/Src pathway which increases the E-cadherin/β-catenin complexes in the adherens junction. Also, Prx2 expression inversely correlates with the metastatic capacity of melanoma cells (106).

Trxs are small 12 kDa redox proteins with a disulfide reducible site. It reduces into Trx-(SH)2 by thioredoxin reductase in the presence of the electron donor, NADPH. Trx isoforms are located in the cytoplasm, nucleus and mitochondria of mammalian cells (107). Trx1 sensitizes melanoma to inhibit glycolytic metabolism which results in inhibition of *in vivo* melanoma metastasis (108). The intracellular expression of Trx and TrxR along with endogenous TNFα expression correlates with the resistance to TNFα-induced cytotoxicity in melanoma cells (109). Melanoma cells secreted Trx induces regulatory T cells (Tregs) infiltration and triggers the survival of Tregs in tumor microenvironment of murine metastatic melanoma model by suppressing antitumor immune response. This mechanism suggests that Trx antibody therapy could be promising for melanoma treatment in clinic (110). ***Non-enzymatic antioxidants*** consist of carotenoids, ascorbic acid (vitamin C), vitamin D derivatives, flavonoids, N-acetyl cysteine (NAC), α-tocopherol (vitamin E), thioredoxin and others antioxidants. Antioxidant supplements administration such as NAC and vitamin E increases melanoma cell invasion and lymph node metastasis in malignant melanoma

murine model (111). Mounting evidences from clinical trial indicate that supplementing diet with antioxidants can increase cancer risks (112). Studies exploring the benefits of antioxidants as dietary supplements failed to correlate that higher intake of antioxidants would lower risk of melanoma development (113, 114). Women receiving vitamin C, vitamin E and ß-carotenoid as dietary supplements demonstrate higher incidence of skin cancer compared to men in a cohort of French population (115). Another study indicates that retinol (a component of vitamin A) supplements in diet might have a significant preventative effect in melanoma among women, however there was no correlation established between melanoma risks with intake of vitamin A (116). Vitamin D3 ($1,25(OH)_2D3$) and novel CYP11A1-derived hydroxyderivatives of D3 show anti-melanoma and protective properties against UVB-induced DNA damage while a defect in the signaling supports melanomagenesis. $1,25(OH)_2D3$ suppresses *in vitro* cell proliferation, colony formation of cultured human and rodent melanoma cell lines. Low levels of $25(OH)_2D3$ is associated with poor patient outcome and advanced melanoma progression. Another vitamin D derivative, 20(OH)D3 attenuates *in vivo* tumor growth of human melanoma cells in immunodeficient mice. Thus inadequate amount of vitamin D, decreased expression of the vitamin D receptors and defects in the enzymes modulating vitamin D activity affect melanomagenesis and tumor progression (117). CYP11A1-derived secosteroids demonstrate protective activity against oxidative stress and UVB-induced DNA damage by attenuating ROS, NO and H_2O_2 generation in keratinocytes, melanocytes, melanoma cells. These derivatives inhibit the generation of cyclobutane pyrimidine dimers in response to UVB radiation, increase phospho-p53 expression at ser-15 position, regulate the GSH level and upregulate the genes encoding enzymes responsible for defense against oxidative stress (118). A phase II trial is initiated in patient with cutaneous melanoma to evaluate the feasibility, safety and toxicity of oral administration of vitamin D (119). Lumisterol, a stereoisomer of ergosterol is produced as a photochemical by-product during the synthesis of vitamin D1. Lumisterol and its hydroxyl derivatives suppress cell proliferation of human skin cells in a cell-type dependent manner along with inhibition of melanoma cell proliferation in both monolayer and soft agar. 20-hydroxylumisterol stimulates the expression of genes associated with keratinocytes differentiation and anti-oxidative process (120). Resveratrol is a naturally occurring polyphenolic antioxidant abundantly present in grapes, red wine and plant extracts. A study found resveratrol exerts its anti-cancer effects by diminishing malignancy of highly invasive B16F10 and BL6 murine melanoma cells. Oral administration of resveratrol suppresses primary tumor volume, AKT activation and lung metastasis in syngeneic melanoma mouse model (121). It induces G1/S cell cycle arrest, inhibits the proliferation of A375 and SK-MEL-31 cells via caspase-3/9 activation, downregulation of Bcl-2 and upregulation of Bax protein expression (122). Resveratrol triggers the autophagy in B16 cells through ceramide accumulation and AKT/mTOR pathway inhibition (123). In combination with ursolic acid (UA) and chloroquine, resveratrol reduces the viability of B16F10 and A375 cells (124). Co-treatment of resveratrol and 5-fluorouracil (5-FU) suppresses cell growth and angiogenesis in B16 tumors (125). Resveratrol is readily absorbed in human and animal models but undergoes rapid metabolism to form sulphate and glucuronide metabolite which lowers its bioavailability to exhibit effective preventive efficacy for melanoma treatment (126-128). Further investigation is required to demonstrate if repeated dosing of the drug can overcome this problem. Melatonin (N-acetyl-5-methoxy tryptamine) is a hormone synthesized by the pineal gland known to regulate circadian rhythmicity and lower vertebrate skin pigmentation (129). It exhibits a broad spectrum of functional properties which include regulation of apoptosis, direct and indirect antioxidative effects, DNA damage repair, immunomodulatory and antitumor activities. Topical application of melatonin and its derivative N^1-acetyl-N^2-formyl-5-methoxykynurenine (AFMK) protect the human and porcine epidermal cells against UVB-induced 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation, DNA damage and cell death with enhancement in $p53^{ser15}$ expression. Melatonin de-monstrates photoprotective action in both pre- and post-UVB treated human and porcine epidermal cells. Melatonin and its derivatives increase the expression of antioxidative enzymes including glutamylcysteine

synthetase (GCS), CAT, glutathione-s-transferase P 1 (GSTP1), GPx and Cu/MnSOD post-UVB irradiation of HaCaT keratinocytes cells. The exogenous application of melatonin or its derivatives prior to UV exposure results in the improved genomic, cellular and tissue integrity against UVB-induced carcinogenesis (130). Melatonin and its metabolites have a modulatory effect on mitochondrion redox and bioenergetic homeostasis (131). Melatonin reduces cell proliferation and induces melanogenesis in SK-MEL-1 cells via p38 MAPK-dependent signaling pathway (132). Melatonin increases the antitumor activity of fisetin (a bio-flavonoid widely present in plants) in MeWo and SK-MEL-28 cells. Melatonin in combination with fisetin induces PARP cleavage, triggers the release of cytochrome-c, enhances the suppression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression, represses the nuclear localization of p300 and NF-κB and abrogates the binding of NF-κB on COX-2 promoter. It also triggers the inhibition of cell proliferation, cell viability and colony formation in combination with fisetin (133). Melatonin differentially suppresses the proliferation of melanoma cell lines (SBCE2, WM-98, WM-164 and SKMEL-188) having specific pattern of melatonin cellular receptor and cytosolic binding protein expression (134).

Other potential ROS targeting therapies in melanoma
Natural therapies from plant source

Dihydromyricetin (DHM) is a flavonoid derived from *Ampelopsisgross dentata* with a broad range of biological and pharmacological properties which exhibits anti-inflammatory, antioxidant and anticancer effects. Treatment with DHM upregulates the expression of autophagic markers (LC3, Beclin-1 and p62) and induces apoptosis *in vitro* via regulating NF-κB signaling mechanism. DHM triggers ROS generation in a time and dose-dependent manner which plays a significant role in DHM-induced NF-κB signaling in human melanoma SK-MEL-28 cells (135). It attenuates melanogenesis by suppressing melanin production and also downregulates MAPK, PKA and PKC signaling cascades (136).

Jacaranone is a benzoquinone isolated from the leaves of *Pentacalia desiderabilis* which induces apoptosis by generating ROS. It has redox cycling capability in which reduced semiquinones formed initiate a signaling cascade that ultimately generates H_2O_2 and hydroxyl free radicals. It demonstrates protective and anti-tumor effects at low therapeutic doses. Jacaranone increases oxidative stress via inducing ROS formation which leads to mitochondrial damage both in *in vitro* and *in vivo* melanoma models. It brings about mitochondrial dysfunction by alteration of the mitochondrial permeability which results in mitochondrial depolarization. Jacaranone treatment significantly increases the levels of pro-apoptotic caspase 2, caspase 3, caspase 8, caspase 9 and Bax in human melanoma A2058 and SK-MEL-28 cell lines. Jacaranone-induced ROS downregulates AKT, activates p38, stimulates apoptosis *in vitro* and delays *in vivo* tumor growth in a dose dependent manner in B16F10 cells (137).

Isoegomaketone (IK) is an essential oil component of *Perilla frutescens* which inhibits *in vivo* tumor growth and induces ROS-mediated apoptosis through mitochondria-dependent/independent pathways in B16 cells. IK-induced apoptosis in these cells is a consequence of upregulation in the Bax/Bcl-2 ratio. It activates the intrinsic caspase 3/9- mediated apoptotic pathway. ROS generation leads to nuclear translocation of apoptosis inducing factor (AIF) which regulates downstream signaling (138). IK-induced ROS generation modulates cell growth inhibition, PI3K/AKT signaling pathway and triggers apoptosis through caspase-dependent and -independent pathways in SK-MEL-2 cells (139). Another anticancer drug, ***celastrol*** suppresses growth and induces apoptosis in B16 melanoma cells via the activation of ROS-mediated caspase-dependent and -independent signaling and the inhibition of of PI3K/AKT pathway (140).

Cryptotanshinone (CT), a diterpene is an active component isolated form *Salvia miltiorrhiza bunge*. It induces apoptosis through the induction of ROS-dependent mitochondrial apoptotic pathway. In addition, it impairs cell proliferation, migration and invasion of A375 cells via downregulation of matrix metallopeptidase-9 (MMP-9) expression. CT triggers ROS-induced apoptosis via increased expression of cleaved caspase 3 and pro-apoptotic protein, Bax along with inhibition of anti-apoptotic Bcl-2 expression (141). Generation of ROS including H_2O_2 and O_2 by CT

boosts the apoptotic effects of TNF-related apoptosis-inducing ligand (TRAIL) *in vitro* and restores the sensitivity of the cells to TRAIL. CT induces the expression of death receptor 5 (DR5) in TRAIL-resistant melanoma cells as a response to ROS-mediated CCAAT/enhancer-binding protein homologous protein (CHOP) activation in A375 cells (142).

Cudraflavone C, a naturally occurring flavonol extracted from the roots of Artocarpus species demonstrates anticancer properties in melanoma cells (143). It induces cellular and mitochondrial ROS generation which results in cellular cytotoxicity and cell cycle arrest in B16 cells. Cudraflavone C stimulates A375.S2 cellular apoptosis via enhancing mitochondrial ROS generation, activating MAPKs (p38, ERK, JNK) and upregulating levels of pro-apoptotic protein (Puma, Bax, Bad, Bid, Apaf-1, cytochrome C, caspase 9 and caspase 3/7) expression (144).

Icariside II (IS), a flavonol glycoside and a metabolite of icariin, derived from *Herba epimedii* exhibits anti-proliferative effect. IS inhibits A375 cell proliferation, induces ROS generation and causes G0/G1 and G2/M cell cycle arrest via activation of p38/p53 pathways along with inhibition of cyclin E, CDK2, cyclin B1 and p-CDK1 expression. NAC abrogates ROS-induced inhibition of A375 cell proliferation and cell cycle arrest (145). IS downregulates cFLIP expression, an anti-apoptotic protein and enhances TRAIL-induced apoptosis via inhibition of ROS-dependent STAT3 and NF-κB signaling. An increase in ROS level activates AKT and decreases phosphorylated STAT3 levels in A375 cells (146).

Curcumin is a primary bioactive component obtained from rhizome of *Curcuma longa* is known to be significantly effective against melanoma growth and progression. Curcumin induces ROS production to activate mammalian STE-20-like kinase 1 (MST1) and induces apoptosis via JNK pathway activation. Additionally, MST1 mediates curcumin-induced Foxo 3a activation and nuclear translocation, where it upregulates Bim-1 and causes cellular apoptosis in B16 and WM-115 cells. ROS scavenger NAC attenuates curcumin-induced JNK and MST1 activation (147). Curcumin alone or in combination with 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) activates JNK signaling and inhibits PI3K/AKT pathway through the production of ROS which in turn mediates DNA damage and apoptosis (148, 149). It prevents proliferation and induces cell death in melanoma cells via regulating cellular redox levels. It induces oxidative stress through ROS production, reducing GSH levels, wrecking mitochondria membrane potential leading to cytochrome C release causing activation of intrinsic cellular apoptosis in A375 cells. All these effects are reversed in presence of NAC as NAC is an anti-oxidant and scavenges the high ROS generated by curcumin treatment. ROS generated in response to curcumin treatment also targets HIF-1α, induces apoptosis via regulating p53 and caspase dependent signaling mechanism (150). Natural borneol synergizes with curcumin to induce apoptosis in A375 cells via inhibition of MAPK/AKT signaling and activation of JNK/caspase-dependent mechanism. It potentiates curcumin to trigger intracellular ROS overburst leading to DNA damage with upregulation of activated p53, ataxia-telangiectasia-mutated (ATM) and pBRCA1 levels (151).

Piperine, an alkaloid obtained from *Piper nigrum* and *Piper longum* demonstrates anti-proliferative effect in repressing the *in vivo* tumor growth using murine melanoma B6 and B16F10 cells (152). Piperine treatment generates ROS which causes cell cycle arrest in SK-MEL-28 and B16F10 cells. Piperine treatment causes DNA damage marked by increase in phosphorylation of ataxia telangiectasia and Rad3-related (ATR) and checkpoint kinase 1 (Chk1) at serine 428 and 296 respectively. Blocking this ROS by tiron protects these cells from piperine-mediated cell cycle arrest in G1 phase and apoptosis (153). It induces apoptosis in A375 and SK-MEL-28 cells via ROS-mediated mitochondrial membrane potential disruption and activation of JNK pathway. This disrupted mitochondrial membrane potential leads to upregulation of cleaved caspase 3, p21, p27 and Bax accompanied with downregulation of Bcl-2 protein expression to mediate apoptosis by intrinsic pathway (154).

Zerumbone, a monocyclic sesquiterpene derived from *Zingiber zerumbet* shows anti-proliferative and anti-migratory effects in CHL-1 melanoma cell line. Treatment with zerumbone increases ROS levels,

decreases mitochondrial matrix potential and reduces mitochondrial transcription factor A mRNA levels. This alters the mitochondrial bioenergetics of these melanoma cells by reduction in the ATP and mitochondrial DNA levels (155).

Jolkinolide B is a bioactive diterpenoid extracted from the roots of *Euphorbia fischeriana steud* which induces ROS production, regulates glycolytic pathway, induces mitochondrial-dependent apoptosis, decreases lactic acid, ATP production in murine melanoma B16F10 cells and suppress *in vivo* tumor growth. Downregulation in the mRNA expression of HK2, lactate dehydrogenase A (LDHA) and GLUT1 promote apoptosis and suppress cell proliferation in response to ROS production. ROS generated in response to Jolkinolide B treatment decreases the mitochondrial membrane potential, upregulates mRNA expression of pro-apoptotic protein such as Bax, downregulates of mRNA levels of anti-apoptotic molecules such as Bcl-2, caspase 3 and caspase 9 (156).

Volatile oil from ginger, obtained from *Zingiber officinale* has an inhibitory effect on cell proliferation and melanogenesis of murine B16 cells. The volatile oil contains various alkene-containing substances (zingiberene and iso-horn teaene) which readily oxidizes and have antioxidant activities to scavenge ROS and inhibit lipid peroxidation. This oil suppresses melanin synthesis and upregulates the levels of antioxidants such as SOD, GSH and CAT in B16 cells. The inhibitory effect of this oil is attributed to its ability to suppress TRP-1, TRP-2, p38 and microphthalmia-associated transcription factor (MITF) activity (157).

Natural therapy from fungal source

Trichodimerol is a secondary metabolite isolated from Trichotechium species, a marine fungus that induces anti-proliferative effect and apoptosis in A375 S2 cells. It causes sub-G1 cell cycle arrest and induces apoptosis via increasing activated caspase 3 and caspase 7 levels. It induces ROS production which mediates the anti-proliferative and pro-apoptotic effects on A375 S2 cells along with activation of p38 and inhibition of ERK (158).

Natural therapy from animal source

Mastoparan is an α-helical, 14-amino acid amphipathic and cationic cell penetrating peptide obtained from venom of wasp *Vespula lewisii*. It demonstrates anti-proliferative effect in a murine model of B16F10-Nex2 cells. It induces oxidative stress by excessive ROS generation in B16F10-Nex2 cells which disrupts the mitochondrial membrane potential and membrane integrity, releases pro-apoptotic molecules into cytosol which leads to activation of caspase-dependent apoptosis. It decreases the expression of Bcl-XL and phospho-Bad (at serine 112) with an increase in the levels of pro-apoptotic proteins such as Bim, Bak, and cytochrome c which triggers apoptosis via intrinsic pathway. Mastoparan also attenuates the growth of subcutaneous melanoma in syngeneic mice and increases their overall survival (159).

ROS targeting therapies from synthetic agents

Disulfiram, a member of the dithiocarbamate family and a copper chelator is currently used for treatment of alcoholism (160). Disulfiram inhibits cell proliferation of M-14, WM-278, WM-1552c melanoma cells, targets spreading of melanoma through superficial and nodular routes via increased ROS production and activates the extrinsic apoptotic pathway (161). It induces apoptosis in A375, C81-46a, c81-61 melanoma cell lines by a redox-associated mechanism, in which it chelates the copper along with depleting and/or oxidizing cellular glutathione to generate oxidative stress which induces apoptosis. Disulfiram augments cell death by reducing the mitochondrial membrane polarization and by decreasing the GSH/GSSG ratio (162, 163). It is tested in phase I/II clinical trial in patients with stage IV melanoma (164). A phase II trial investigating the benefits of co-administration of disulfiram with chelated zinc for patients with refractory disseminated malignant melanoma who have failed to show a response to first line therapy has been completed (165).

Choline tetrathiomolybdate (ATN-224) is a second generation ammonium tetrathiomolybdate analogue with a high copper binding affinity that inhibits SOD1. Inhibition of SOD1 in tumor cells attenuates angiogenesis, cancer cell proliferation in *in vitro* and *in vivo* melanoma models by various pathways such as

inhibition of VEGF, induction of p-ERK and signaling molecules as PKB/AKT and NF-κB expression (166, 167). ATN-224 in combination with a DNA alkylating agent, temozolomide demonstrates additive cytotoxicity in five melanoma cell lines including M14, WM3211, YUZAZ6, SK-Mel-5 and A375. ATN-224 synergizes with buthionine sulfoximine to show its effect *in vitro*, where as it demonstrates moderate antagonistic effect with arsenic trioxide or disulfiram, both of which are known to interfere with glutathione recycling (168). It reduces serum copper levels in phase I clinical trial in patient with metastatic melanoma with a recommended phase II dose of 300 mg/day (169). A phase II trial evaluating the safety and efficacy of ATN-224 in combination with temozolomide for patients with advance melanoma has been conducted (170).

Elesclomol is identified from a phenotypic screening of small molecules displaying potent pro-apoptotic activities. Concomitant treatment of elesclomol increases intracellular ROS content and HSP70 RNA levels in Hs294T melanoma cells. Also, elesclomol induces apoptosis via oxidative stress induced cytochrome c release and caspase 3-dependent pathways. This effect is nullified by NAC in Hs294T cells (171). Generation of ROS by elesclomol is in part dependent on chelation and redox recycling of copper in the mitochondria via electron transport system. Elesclomol chelates copper outside of cells leads to elesclomol-Cu II complex formation. This complex selectively transports the copper to mitochondria where Cu II is reduced to Cu I, followed by subsequent ROS production (172, 173). A phase III trial investigating the combination of elesclomol with paclitaxel in patients with advanced melanoma has been terminated as the combination did not improve the progression free survival (PFS) significantly compared to paclitaxel single agent (174, 175). A phase III trial exploring the advantage of combination of elesclomol with paclitaxel vs paclitaxel alone in stage IV metastatic melanoma patients showed minimal improvement in the PFS without any statistical significance (176).

SC-514 is an IKKβ inhibitor which induces ROS generation. Increased ROS levels result in enhanced DNA crosslinking efficiency triggered by fotemustine, an alkylating agent and nitrosourea family member. SC-514 enhances ATM phosphorylation and sensitizes fotemustine-induced cell death in A375, G361, A2058, SK-MEL2, SK-MEL-5, SK-MEL-28, Hs294T, IGR-1, MeWo, Colo829, Malma-3M melanoma cell lines. SC-514 synergizes with fotemustine to reduce tumor size and malignancy *in vivo* (177).

TRAM-34 is clotrimazole analog and selective calcium (Ca^{2+})-dependent potassium (K^+) channel inhibitor (KCa3.1). This sensitizes A375 cells to vemurafenib-induced cell death via caspase 3-dependent signaling activation. This synergistic combination alters the mitochondrial membrane potential, increases intracellular ROS levels and is effective in vemurafenib-resistant cells. The antioxidant vitamin E overcomes the effect of activated caspase signaling and ROS production (178). TRAM-34 also increases TRAIL-induced apoptosis via controlled release of second mitochondria-derived activator of caspases (SMAC) and anti-apoptotic cellular inhibitors of apoptosis protein (cIAP) (179).

Zinc induces apoptosis by increasing ROS levels and via modulation of p53 and FAS/FAS ligand (FASL) protein expression in WM 266-4 cells. p53 regulates the intracellular redox state and induces ROS-dependent apoptosis (180). Zinc oxide nanoparticles known to possess semiconductor properties induce oxidative stress, enhance ROS production and deplete GSH in A375 cells. These particles induce genotoxic and apoptotic response via caspase 3-dependent mechanism (181). ZnO nanoparticles also induce oxidative stress in Cloudman S91 melanoma cancer cells. Exposure to these nanoparticles leads to a spontaneous increase in the ROS levels and membrane lipid peroxidation along with a decrease in the level of GSH, SOD and catalase (182).

AC-1001 H3 CDR peptide is a murine monoclonal antibody-derived peptide in which the heavy chain complementary determining region (V_H CDR3) on immunoglobulin domain inhibits lung metastasis in a syngeneic mice model generated using B16F10-Nex2 cells. It alters the mitochondrial membrane potential and induces ROS production. This ROS induces cytotoxic effect *in vitro* in human A2058 and murine B16F10 melanoma cells via activation of intrinsic apoptotic pathway. It increases expression of

LC3/LC3II along with Beclin1 which are early signs of autophagy (183).

Di-methyl-ampal-thio-ester (DIMATE) is an isoform specific competitive irreversible inhibitor of aldehyde dehydrogenase (ALDH) isoforms 1 and 3. Epigenetic mechanism upregulates ALDH1A3 significantly in melanoma cells versus normal melanocytes. Elevated levels of ALDH1 activity correlates with increased ROS levels in several melanoma and patient-derived cell lines. Elevated levels of ROS in turn cause generation and accumulation of apoptogenic aldehydes such as 4-hydroxynoneal (4-HNE) and malondialdehyde (MDA) ultimately leading to cellular apoptosis marked by increased Bax and reduced Bcl-2 levels. DIMATE reduces *in vivo* tumor growth and targets slow cycling patient-derived cell population consisting of the tumorigenic and chemo-resistant melanoma tumor cells (184).

PD-0332991 (Palbociclib), a CDK4/6 inhibitor enhances TRAIL sensitivity via inducing ROS generation. TRAIL sensitivity correlates positively with the induced cell cycle arrest in TRAIL-sensitive/resistant or partially acquired resistant melanoma cell lines. In addition to cell cycle arrest, apoptosis is induced in these cells due to loss of mitochondrial membrane potential, ROS production, upregulation of Bcl-2 and PUMA levels in response to combined treatment of PD-0332991 and TRAIL (185).

Cerium oxide (CeO_2) nanoparticles enter the general circulation and reach the distant tissues/organs readily. These particles induce ROS production which leads to DNA damage, apoptosis via ROS-triggered mitochondrial pathway marked by an increase in caspase 3 levels, chromosomal condensation and fragmentation *in vitro*. This nanoparticle possesses genotoxic property that stimulates single and double strand DNA breaks (186, 187). Co-administration of nanoparticles with doxorubicin enhances anti-tumor property of doxorubicin by increasing the cytotoxicity and ROS formation leading to oxidative damage in A375 cells (188). A375 cells treated CeO_2 alters the intracellular redox status of the cells in response to a surge in the levels of ROS. This leads to activation of the apoptotic pathway by releasing cytochrome c, activating caspase 3 and inducing PARP cleavage. It also leads to decrease in tumor growth in *in vivo* mice model (189).

Lomefloxacin, a fluoroquinolone and a topoisomerase II inhibitor is a synthetic antibiotic used to treat infections. Treatment of COLO829 melanoma cells with this drug leads to overproduction of ROS and depletes the level of intracellular glutathione. Overproduction of ROS disturbs the intracellular redox balance thus inducing oxidative stress leading to oligonucleosomal DNA fragmentation. Loss of mitochondrial membrane potential causes S, G2/M cell cycle arrest and apoptosis (190).

Vorinostat, a histone deacetylate inhibitor is effective against BRAF-inhibitors or BRAF plus MEK inhibitors resistance in *in vitro*, *in vivo* and clinical studies against melanoma. Treatment with vorinostat suppresses SLC7A11 gene (gene encoding for a precursor of ROS scavenger glutathione) which adds to elevated ROS levels and induces DNA damage and cell death (191).

Other potential ROS-targeting agents in melanoma

Calpain 3 (p94), a gene product of *CAPN3* belongs to superfamily of calcium-regulated intracellular cysteine proteases is predominantly expressed in skeletal muscles. Melanoma tissues express higher CAPN3 compared to other tumor types (192). Calpain 3 induces ROS production *in vitro* and p53 plays a vital role in its regulation. It increases the expression of oxidative stress-related gene, RANTES (codes for CCL5 chemokine) which impairs cell proliferation and induces cell death in HT-144 and A375 cells. Active calpain-3 (variant hmp84) leads to accumulation of p53, modulates oxidative stress and induces DNA damage (193).

Recent study indicate that aged fibroblasts secret a Wnt antagonist, ***sFRP2*** which regulate multiple signaling mechanism in melanoma cells that decreases β-catenin and MITF levels and leads to the loss of a key redox effector, APE1. Loss of APE1 expression inhibits the response of melanoma cells to ROS-induced DNA damage and increases the resistance of melanoma cells to BRAF-targeted therapies. Hence, sFRP2 could be a promising target in ROS-mediated signaling for treatment of metastatic melanoma (194).

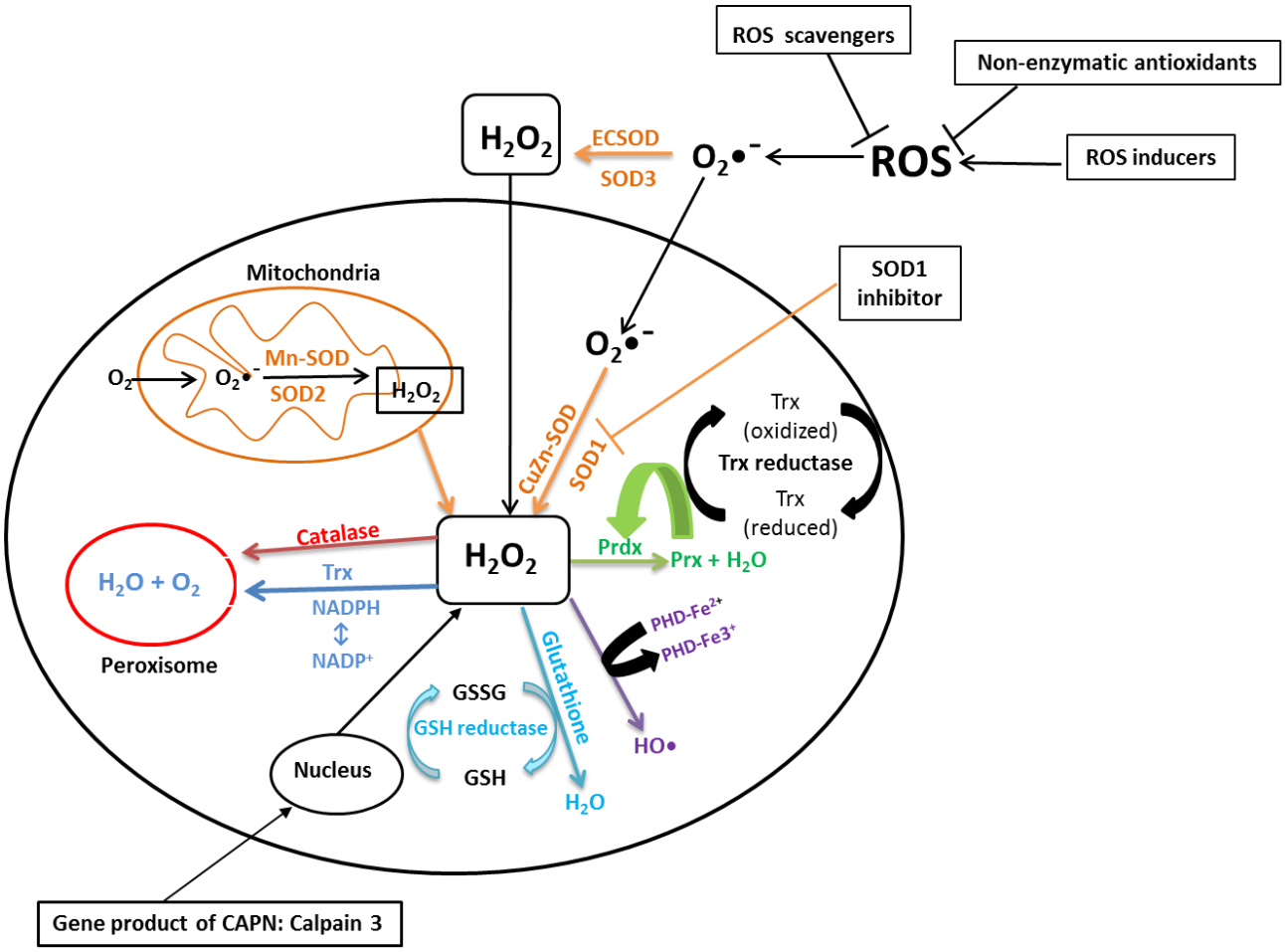

Figure 2. ROS production and targeted therapies. Prdx: Reduced peroxiredoxin; Prx: Oxidized peroxiredoxin; GSH: Reduced glutathione; GSSG: Oxidized glutathione; Trx: Thioredoxin; ECSOD: Extracellular superoxide dismutase; SOD: Superoxide dismutase; NADPH: Nicotinamide adenine dinucleotide phosphate. ROS targeting agents includes: ROS scavenger (Volatile oil from ginger); Non-enzymatic antioxidants (Vitamin C, Vitamin E, ß-carotene, Retinol, Vitamin D3 and its derivatives, Lumisterol and its derivatives, Resveratrol alone or in combination with Ursolic acid and Chloroquinone/5- Flurouracil, Melatonin alone or in combination with Fisetin) and enzymatic anti-oxidants (Superoxide dismutase; Catalases and peroxidases). ROS inducers obtained from natural source includes Flavonoid: Dihydromyricetin; Benzoquinone: Jacaranone; Essential oil: Isoegomaketone; Celastrol; Diterpene: Cryptotanshinone; Flavonol: Cudraflavone C; Flavonol glycoside: Icariside II; Rhizome: Curcumin alone or in combination with 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and Borneol; Alkaloid: Piperine; Monosyclic sesquiterpene: Zerumbone; Diterpenoid: Jolkinolide B; Fungal source: Trichodimerol; Animal source: Mastoparan. Synthetic agents that induce ROS include: Copper chelator: Disulfiram; Elesclomol; IKKβ (Inhibitor of nuclear factor kappa-B kinase subunit beta) inhibitor: SC-514; TRAM-34 alone or in combination with vemurafenib; Zinc and Zinc oxide nanoparticles; Murine momoclonal antibody-derived peptide: AC-1001 H3 CDR; Aldehyde dehydrogenase inhibitor: DIMATE; Cyclin dependent kinase 4/6 inhibitor: PD-0332991; Cerium oxide nanoparticles alone or in combination with Doxorubicin; Fluoroquinolone antibiotic: Lomefloxacin; Histone deacetylase inhibitor: Vorinostat; Wnt antagonist: sFRP2. SOD1 inhibitor (ATN-224 alone or in combination with Buthionine sulfoximine (BSO)/arsenic trioxide/temozolamide/disulfiram.

To summarize, five drugs including vitamin D and its derivatives, disulfiram, ATN-224, ATN-224 in combination with temozolomide and elesclomol have undergone various stages of clinical trial. In addition,

preliminary clinical studies have been performed using retinol and vitamin C in combination with vitamin E and ß-carotene in patient with metastatic melanoma. Table 1 summarizes ROS targeted therapies and the associated signaling pathways. Figure 2 summarizes the ROS production and the targeted therapies.

Conclusion and future prospectus

Oxidative stress and ROS are crucial etiological factors for melanoma progression. The pleiotrophic effects of ROS induce diverse cellular responses that contribute to melanoma progression. Two main therapeutic approaches have been exploited to regulate ROS levels to control oxidative stress and cancer cell metastasis. One strategy was to interrupt ROS-mediated signaling cascade and cancer metastasis via antioxidant based ROS scavenging mechanism. However, single antioxidant therapy failed in clinical testing owing to increase tumor development. Also, endogenous antioxidants synthesized in cancer cells including melanoma as a result of adaptive response to increased ROS ultimately mask the effect of exogenous antioxidants. Another approach used was to induce ROS production in the cancer cells and to inhibit the cellular antioxidant levels. In preclinical studies, this approach resulted in cytotoxicity of the cancer cells with increased endogenous ROS production. Most cancer cells adapt to altered redox mechanism induced by ROS generating agents and eventually develop resistance. A combinatorial approach of epigenetic therapy with drugs that are capable of preventing generation and chronic accumulation of ROS along with standard chemotherapeutic regimens or radiotherapy might be crucial in overriding intrinsic melanoma resistance. Another recent approach is the use of drugs which induce ROS accumulation in melanoma cells to trigger ferroptosis, an iron-dependent form of regulated non-apoptotic cell death. Several studies are ongoing to explore combinatorial approach of ferroptosis-inducing drugs with conventional immunotherapy or kinase inhibitors to overcome resistance in de-differentiating melanoma cells. Exploring the benefits of these prospective therapies for treatment of patients with metastatic melanoma requires further investigation.

Acknowledgements

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Table 1: Summary of ROS-targeted therapies and associated signaling in melanoma

Therapy	Type	Mechanism /signaling pathways affected	Stage
Antioxidant enzymes and therapies			
SOD1,SOD2,SOD3	Enzymatic antioxidant	Converts $O_2\bullet^-$ to H_2O_2 [97]. Inhibits vemurafenib-induced upregulation of intracellular $O_2\bullet^-$ and NO production [99].	Preclinical
CAT	Enzymatic antioxidant	Decomposes H_2O_2 to H_2O and O_2. PEG-CAT suppresses melanoma growth [101, 102], reduces EGF and EGFR levels [102].	Preclinical
Glutathione	Enzymatic antioxidant	Reduces H_2O_2 to H_2O [103] and shows protective effects against oxidative stress [104].	Preclinical
Peroxiredoxin	Enzymatic antioxidant	Reduces H_2O_2 to H_2O [105]. Regulates ERK/Src pathway and inhibits metastasis [106].	Preclinical
Trx	Enzymatic antioxidant	Reduced to Trx-(SH)2 by thioredoxin reductase, inhibits glycolytic metabolism [107] and metastasis [108], induces Tregs infiltration [110].	Preclinical
Vitamin E and NAC	Non-enzymatic antioxidant	Increases melanoma cell invasion and lymph node metastasis in *in vivo* model [111].	Preclinical
Vitamin C, vitamin E and β-carotene	Non-enzymatic antioxidant	Increases incidence of skin cancer in combination [115].	Clinical
Retinol	Non-enzymatic antioxidant	Shows significant preventive effect in melanoma [116].	Clinical
Vitamin D3 and its hydroxyderivatives	Non-enzymatic antioxidant	Protection against DNA damage [117], attenuate ROS signaling and upregulate genes encoding enzymes for defense against oxidative stress [118].	Preclinical and clinical: Phase II [119]
Lumisterol and its hydroxyderivatives	Non-enzymatic antioxidant	Stimulate genes associated with keratinocyte differentiation anti-oxidative process [120].	Preclinical
Resveratrol	Polyphenolic antioxidant	Activates AKT, inhibits *in vivo* tumor growth [121], induces cell cycle arrest and autophagy [122,123].	Preclinical
Resveratrol plus UA and chloroquine	Polyphenolic antioxidant	Inhibits cell proliferation, reduces autophagosome levels , increases LC3II and decreases Beclin-1/p62 levels [124].	Preclinical
Resveratrol plus 5-FU	Polyphenolic antioxidant	Upregulates p-AMPK and downregulates COX-2, VASP and VEGF levels [125].	Preclinical
Melatonin	Hormone synthesized by pineal gland	Melatonin and AFMK protects against DNA damage [130], modulates redox signaling [131] and induces melanomagenesis [132].	Preclinical
Melatonin in combination with fisetin	Hormone plus a bio-flavonoid	Triggers apoptosis, suppresses COX-2 and iNOS expression and inhibits binding of NF-κB on COX-2 promoter [133].	Preclinical
Natural therapies from plant source			
Dihydromyricetin	ROS-inducing flavonoid	Upregulates autophagy markers, induces apoptosis [135], downregulates PKA, MAPK and PKC pathways [136].	Preclinical
Jacaranone	ROS-inducing benzoquinone	Increases oxidative stress, induces apoptosis, inhibits AKT and upregulates p38 [137].	Preclinical
Isoegomaketone	ROS-inducing essential oil component	Induces cell death via caspase-dependent pathways and modulates PI3K/AKT [138,139].	Preclinical
Celastrol	ROS-inducer	Induces apoptosis and inhibits PI3K/AKT signaling [140].	Preclinical

Cryptotanshinone	ROS-inducing diterpene	Induces apoptosis [141], boosts apoptotic effects of TRAIL and induces DR5 expression in TRAIL-resistant melanoma cell lines [142].	Preclinical
Cudraflavone C	ROS-inducing flavonol	Activates MAPKs and induces apoptosis [144].	Preclinical
Icariside II	ROS-inducing flavonol glycoside	Induces cell cycle arrest [145], inhibits ROS-dependent STAT3 and NK-κB signaling [146].	Preclinical
Curcumin	ROS inducer	Induces apoptosis via JNK [147] and p53-dependent signaling [150].	Preclinical
Curcumin alone or with PDMP	ROS inducer	Activates JNK and inhibits PI3K/AKT signaling pathways [148,149].	Preclinical
Curcumin with natural borneol	ROS inducer	Inhibits MAPK/AKT and activates JNK/caspase-dependent mechanism [151].	Preclinical
Piperine	ROS-inducing alkaloid	Represses tumor growth [152], causes DNA damage [153] and induces apoptosis via activating JNK pathway [154].	Preclinical
Zerumbone	ROS-inducing monocyclic sesquiterpene	Alters MMP which reduces mitochondrial transcription factor A mRNA, ATP and mitochondrial DNA levels [155].	Preclinical
Jolkinolide B	ROS-inducing diterpenoid	Induces mitochondrial-dependent apoptosis and affects glycolytic signaling [156].	Preclinical
Volatile oil form ginger	ROS scavenger containing oxidized alkene substance	Attenuates melanin synthesis, increases antioxidants, suppresses TRP-1, TRP-2, p38 and MITF expression [157].	Preclinical
Natural Therapies from marine fungal source			
Trichodimerol	ROS-inducing secondary metabolite	Induces cell cycle arrest, activates p38 and inhibits ERK signaling [158].	Preclinical
Natural Therapies from animal source			
Mastoparan	ROS-inducing peptide	Activates intrinsic apoptosis pathway [159].	Preclinical
Synthetic agents			
Disulfiram	ROS-inducing copper chelator	Activates extrinsic apoptosis pathway [161], decreases GSG/GSSG ratio [162,163].	Preclinical and clinical: Phase I/II [164-165]
Choline tetrathiomolybdate (ATN-224)	SOD1 inhibitor	Attenuates angiogenesis and regulates MAPK, AKT and NF-κB signaling [166,167].	Preclinical and clinical: Phase I [169]
ATN-224 with temozolomide /BSO/arsenic trioxide/disulfiram	SOD1 inhibitor in combination with other therapies	Exhibits additive cytotoxicity *in vitro* with temozolamide, synergizes with BSO and shows moderate antagonistic activity with arsenic trioxide and disulfiram [168].	Preclinical and clinical: Phase II [170]
Elesclomol	ROS inducer	Triggers apoptosis [171] and effects redox signaling [172,173].	Preclinical and clinical: Phase III [174-176]
SC-514	ROS-inducing IKKß inhibitor	Enhances DNA crosslinking efficiency of fotemustine, induces p-ATM and sensitizes fotemustine-induced cell death [177].	Preclinical
TRAM-34 in combination with vemurafenib	Selective calcium-dependent potassium channel inhibitor and BRAF inhibitor	Induces caspase 3-dependent apoptotic pathway [178].	Preclinical

TRAM-34	Selective calcium-dependent potassium channel inhibitor	Increases TRAIL-induced apoptosis via release of SMAC and cIAP [179].	Preclinical
Zinc	ROS inducer	Modulate p53 and FASL protein expression [180].	Preclinical
Zinc oxide nanoparticles	ROS inducer	Deplete antioxidant levels and induces apoptosis [181] and increases membrane lipid peroxidation [182].	Preclinical
AC-1001 H3 CDR peptide	ROS-inducing murine mAb-derived peptide	Activates intrinsic apoptotic pathway and shows early signs of autophagy [183].	Preclinical
DIMATE	ROS-inducing competitive irreversible inhibitor of ALDHA 1/3	Leads to accumulation of HNE and MDA which causes apoptosis [184].	Preclinical
PD-0332991	ROS-inducing CDK4/6 inhibitor	Enhances TRAIL sensitivity, induces cell cycle arrest and apoptosis in combination with TRAIL [185].	Preclinical
Cerium oxide nanoparticles	ROS inducers	Causes DNA damage and apoptosis [186,187], decreases *in vivo* tumor growth [189].	Preclinical
Cerium oxide nanoparticles with doxorubicin	ROS inducers	Causes oxidative damage, alters the intracellular redox status and increases cellular cytotoxicity [188].	Preclinical
Lomefloxacin	ROS-inducing fluoroquinolone antibiotic	Decrease the levels of intracellular GSH, causes oligonucleosomal fragmentation, cell cycle arrest and apoptosis [190].	Preclinical
Vorinostat	HDAC inhibitor	Suppresses SLC7A11 [191].	Preclinical
Miscellaneous therapies			
Calpain 3	ROS-inducing gene product of CAPN3	Increases expression of RANTES [192], activated calpain 3 leads to accumulation of p53 and induces DNA damage [193].	Preclinical
sFRP2	Wnt antagonist	Regulates APE1 expression [194].	Preclinical

Abbreviations: 5-FU: 5-Fluorouracil; AFMK: N^1-acetyl-N^2-formyl-5-methoxykynurenine; ALDH: Aldehyde dehydrogenase; AMPK: Adenosine monophosphate-activated protein kinase; APE1: apurinic/apyrimidinic endonuclease 1; ATM: Ataxia-telangiectasia-mutated; BSO: Buthionine sulfoximine; CAT: Catalase; cIAP: Cellular inhibitor of apoptosis protein; CDK: Cyclin dependent kinase; COX 2: Cyclooxygenase 2; DIMATE: Di-methyl-ampal-thio-ester; DR5: Death receptor 5; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; FASL: FAS/FAS ligand; GSH: Reduced glutathione; GSSG: Oxidized glutathione; HDAC: Histone deacetylase; HNE: 4-hydroxynoneal; IKKβ: inhibitor of nuclear factor kappa-B kinase subunit beta; iNOS: Inhibitor of nitric oxide synthase; JNK: c-Jun N-terminal kinase; LC3: Microtubule-associated protein 1A/1B-light chain 3; mAb: monoclonal antibody MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; MITF: Microphthalmia-associated transcription factor; MMP: Mitochondrial membrane potential; NAC: N-acetyl cysteine; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NO: Nitric oxide; PDMP: 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PEG: Polyethylene glycol; PKA: Protein kinase A; PKC: Protein kinase C; RANTES: Regulated on activation, normal T cell expressed and secreted; ROS: Reactive oxygen species; sFRP2: Secreted frizzled- related protein 2; SMAC: second mitochondria-derived activator of caspases; SOD: Superoxide dismutase; STAT3: Signal transducer and activator of transcription 3; TRAIL: TNF-related apoptosis-inducing ligand; TRP: Tyrosinase related protein; Trx: Thioredoxin; Treg: Regulatory T-cells; UA: Ursolic Acid; VASP: Vasodilator-stimulated phosphoprotein; VEGF: Vascular endothelial growth factor.

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