ROS

REVIEW ARTICLES

ROS-Inducing Agents for Cancer Chemotherapy

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ABSTRACT | Reactive oxygen species (ROS) play an essential role in maintaining cellular homeostasis, and levels of ROS are regulated by redox enzymes and reduced factors such as glutathione. Excess levels of ROS can result in DNA and cellular damage which can contribute to development of tumors. Cancer cells exhibit increased metabolic activity and ROS levels compared to normal cells and, with threshold limits, ROS contribute to cancer cell homeostasis and growth. However, treatment of cancer cells with ROS-inducing anticancer agents exceeds the threshold for ROS and this results in activation of multiple cell death pathways which include inhibition of mammalian target of rapamycin (mTOR) signaling and downregulation of specificity protein (Sp) transcription factors Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes. Thus, ROS-inducing drugs represent a highly effective group of mechanism-based agents for individual and combined cancer chemotherapies.

KEYWORDS | Antineoplastic activity; Reactive oxygen species

ABBREVIATIONS | AMPK, AMP-activated protein kinase; ANT, adenine nucleotide translocase; CDDO-Me, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate; DNMT1, DNA methyltransferase 1; GPx, glutathione peroxidase; GSH, glutathione; HIF-1, hypoxia inducible factor 1; JNK, c-jun N-terminal kinase; LKB1, liver kinase B1; MMP, mitochondrial membrane potential; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; PEITC, phenethylisothiocyanate; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; RNS, reactive nitrogen species; ROS, reactive oxygen species; SIRT1, histone deacetylase sirtuin-1; SOD, superoxide dismutase; Sp, specificity protein; TSC, tuberous sclerosis complex

CONTENTS

1. DIFFERENT REACTIVE OXYGEN SPECIES/REACTIVE NITROGEN SPECIES

Reactive oxygen species (ROS) are emerging molecules or ions formed by one or more unpaired electrons of oxygen [1]. The unpaired electrons of oxygen react to form partially reduced highly reactive species that are classified into two groups: free radical and non-radical oxygen species. Oxygen free radicals include superoxide anions ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$), organic radicals ($R^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$), thiyl radicals ($RS^{\bullet}$), sulfonyl radicals ($ROS^{\bullet}$), and thiyl peroxyl radicals ($RSOO^{\bullet}$). Non-radical ROS include hydrogen peroxide (H_2O_2), delta state singlet oxygen ($^1O_2$), ozone (also known as trioxygen) (O_3), organic hydroperoxides (ROOH), and hypochlorous acid (HOCl).

Reactive nitrogen species (RNS) are a variety of nitrogen containing molecules that are typically derived from nitric oxide reactions. Nitric oxide chemically combines with superoxide by an enzyme-independent mechanism to form peroxynitrite ($ONOO^-$), a strong oxidant that reacts with most biological molecules, causing cell damage. Nitric oxide and peroxynitrite are not the only RNS, and RNS also include nitroxyl (NO^-), nitrosonium cation (NO^+), higher oxides of nitrogen, S-nitrosothiols (RSNOs), and dinitrosyl iron complexes [2].

2. SOURCES OF ROS/RNS

ROS/RNS can be produced from endogenous and some exogenous sources, including pollutants, tobacco smoke, and radiation, and ROS/RNS generated during different cellular reactions may be either favorable or harmful to the cells. Cellular ROS are produced from various enzyme systems, including the mitochondrial electron transport chain, cytochrome P450 enzymes, lipoxygenases, cyclooxygenases, the NADPH oxidase complex, xanthine oxioxidase, enzymes in peroxisomes, and thymidine phosphorylase [3]. Among them, the mitochondrial electron transport chain is the major source of intracellular ROS generation, and it is estimated that 3–5% of oxygen consumed is ultimately converted towards ROS production in isolated mitochondria [4]. The mitochondrial electron transport chain contains enzyme complexes I, II, III, and IV, and complexes I, III, and IV utilize the free energy released by a series of spontaneous redox reactions to generate a proton electrochemical gradient across the mitochondrial inner membrane. Superoxide generation occurs in the mitochondrial inner membrane by a non-enzymatic, single-electron transfer to molecular dioxygen by ubisemiquinone in complex III and by reduced flavin mononucleotide in the NADH dehydrogenase complex [5–7]. In the outer membrane of the mitochondria, the mitochondrial permeability transition pore allows the leakage of superoxide into the cytoplasm [8] and nucleus [9]. Almost all cells contain enzymatic antioxidant defense mechanisms that rapidly metabolize ROS. Specifically, superoxide is dismutated by superoxide dismutase (SOD) with either copper/zinc (Cu/Zn) or manganese (Mn) metal centers that catalyze the oxidation and reduction of superoxide to form O_2 and H_2O_2 in a reaction that is effectively diffusion limited [10, 11]. Cu,ZnSOD is present in the cytosol, while MnSOD is found in the mitochondria. H_2O_2 is consequently converted to water by either catalase [12] or glutathione peroxidase (GPx) [13].

Moreover, RNS are also produced within mitochondria, as the inducible form of nitric oxide synthase (NOS) catalyzes the formation of $NO^{\bullet}$ and L-citrulline from L-arginine and oxygen via a 5-electron redox reaction [14]. Nitric oxide can revers-

ibly inhibit cytochrome c oxidase and increase the reduced state of electron carriers in the respiratory chain, resulting in $O_2^{\bullet -}$ production. A product of $NO^{\bullet}$ oxidation, namely, nitrogen dioxide radical ($NO_2^{\bullet}$), can oxidize or nitrate a wide range of biomolecules. Peroxynitrite can oxidize thiol groups, DNA bases, and tyrosine residues. In mitochondria, excessive $ONOO^-$ levels can impair oxidative phosphorylation by inhibiting complex I, complex IV, ATP synthase, and MnSOD activity, as well as disrupting calcium homeostasis [15].

In addition to the mitochondrial electron transport chain, peroxisomes are another potential source of ROS generation and scavenging. Peroxisomes are versatile organelles involved in fatty acid oxidation, catabolism of purine, and biosynthesis of glycerolipids and bile acids [16]. As mitochondria and peroxisomes are closely linked, metabolic tasks of peroxisomes like β-oxidation or amino acid metabolism are accomplished in cooperation with mitochondria [17]. Peroxisomal oxidases catalyze the oxidative breakdown of different fatty acids, purines, amino acids, polyamines, and α-hydroxy acids, which is followed by transferring the hydrogen extracted from the appropriate substrate directly to O_2, forming H_2O_2. The H_2O_2 produced from peroxisomal oxidases is subsequently converted to H_2O and O_2 by catalase, the most prominent enzyme of peroxisomes. Further, xanthine oxidase, an enzyme involved in the catabolism of purine, also generates $O_2^{\bullet -}$ and H_2O_2 in both the matrix and the membranes of peroxisomes. Xanthine oxidase is a molybdenum-containing dimeric flavoenzyme and functionally exists in two forms: the NAD^+-dependent D- or dehydrogenase form and the O-type, which reduces O_2 and hence has to be considered an oxidase. The activity of xanthine oxidase was determined exclusively in the core fraction of the purified rat hepatic peroxisomes [18].

3. ROLE OF ROS IN MAINTAINING CELLULAR HOMEOSTASIS

ROS are essential for living organisms and their biological functions. ROS play an important role in physiological conditions, including: regulation of cell signaling, cell growth, apoptosis, differentiation; and activity of several enzymes; stimulation of cytokine production; and elimination of pathogens and foreign particles. Numerous studies show that a large number of intracellular signaling pathways are regulated by intracellular ROS (reviewed in Ref. [19]). Multiple growth factors and cytokines that bind to cell membrane receptors, including cytokine receptors, receptor tyrosine kinases, receptor serine/threonine kinases, as well as G protein-coupled receptors, stimulate ROS production. Further, it has been reported that ROS activate mitogen-activated protein (MAP) kinase/Erk cascade, phosphoinositide 3-kinase (PI3K)/Akt-regulated signaling cascades, and IκB kinase (IKK)/nuclear factor κB (NF-κB)-activating pathways [20, 21].

In addition to the activation of various signaling cascades involved in cell growth and differentiation, ROS may directly regulate the activity of transcription factors through oxidative modifications. Several transcription factors have been shown to be redox-sensitive, including NF-κB, activator protein (AP)-1, specificity protein (Sp)-1, c-Myb, p53, early growth response (egr)-1, and hypoxia inducible factor (HIF)-1α [14]. Studies suggest that responses of cells to cytokines and growth factors are dependent on the cell redox status. The redox status results from a subtle equilibrium between ROS production and intracellular antioxidants levels. This balance is slightly modulated by exogenous factors, such as oxygen tension or cytokines.

Intracellular levels of ROS are maintained within defined ranges to prevent cell damage and maintain homeostasis. However, when ROS overcome the cellular antioxidant defense system and antioxidant capacity, then oxidative stress occurs, resulting in damage of lipids, proteins, and DNA [22]. These hallmarks of oxidative stress have been implicated in many pathological conditions such as carcinogenesis [23], aging [24], neurodegeneration [25, 26], and diabetes mellitus [27].

4. ROLE OF ROS IN CANCER CELLS/TUMORS

4.1. Sources

Dysfunction of mitochondria, activation of cell signaling, oncogenes, aberrant metabolism, increased activities of oxidases, cyclooxygenases, lipoxygenases, and loss of functional p53 are known to increase the production of ROS in cancer cells [28–

30]. Several growth factors and cytokines also increase ROS production [31, 32]. In response to interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), the levels of H_2O_2 and nitric oxide are increased in tumor cells [17, 18]. Oncogenes, such as Ras, Bcr-Abl, c-Myc, and c-Met, also induce ROS production [33, 34]. For instance, K-Ras and its oncogenic mutations have been tightly associated with increased generation of $O_2^{\bullet-}$ through the activation of the membrane-associated ROS-producing enzyme NADPH oxidase in various cancers [35–37]. c-Met increases the generation of $O_2^{\bullet-}$ by the activation of NADPH oxidase through Rac-1 [28], and active Rac-1 induces H_2O_2 production through the activity of 5-lipoxygenase [38]. Moreover, high levels of ROS can result from suppression of antioxidant molecule sestrin 1 (SeSN1) by the activation of Ras oncogenic signaling [39]. Studies suggest that ROS also increase mitochondrial DNA mutations in various cancer cells [40, 41].

4.2. ROS Levels in Cancer Cells versus Normal Cells

ROS are inevitably generated through cellular metabolism and redox regulation in cancer cells, which tend to have increased levels of endogenous ROS compared to normal cells [42, 43]. Increased oxidative stress due to persistent pro-oxidative state is a main feature of cancer cells, and high levels of ROS and lipid peroxidation in cancer cells are correlated with decreases in enzymatic and non-enzymatic antioxidants, including SOD, catalase, vitamin C, and glutathione [44, 45]. High levels of ROS in cancer cells are due to the byproducts of increased metabolic activity of the cells, and these levels are important for the function of cancer cells [43]. However, these cells are also more vulnerable to ROS-induced cytotoxicity. For example, cells expressing oncogenic Ras [46] and hyperactive PI3K/Akt signaling [47] exhibit increased susceptibility to oxidative stress-induced cell death.

4.3. Functions of ROS in Cancer Cells and an Upper Threshold

In the past two decades, oxidative stress-mediated cancer promotion and progression have been linked to increasing DNA mutations or DNA damage, genome instability, cell cycle progression, cell survival and disruption of cell death signaling, epithelial-mesenchymal transition and metastasis, cell-cell adhesion, angiogenesis, and regulation of cancer stem cells (see detailed review in [43]). For instance, cell proliferation and quiescence are regulated by mitochondria-derived ROS. Low H_2O_2 levels stimulate cell proliferation due to decreased MnSOD activity, whereas increased production of H_2O_2 drives the proliferating cells into quiescence, due to increased MnSOD activity [48]. Moreover, the highly invasive pancreatic and metastatic breast cancer cells show low levels of H_2O_2 and increased activity of MnSOD, suggesting that redox regulation is important for the cancer metastatic process [49, 50]. Hypoxia contributes to the malignant phenotype and aggressive tumor progression in various tumor types by inducing several transcription factors, including HIF-1 [51]. Increased ROS levels were found to activate HIF-1 signaling to increase tumor progression [52]. Multiple studies suggest a role for ROS in increasing angiogenesis. For example, angiogenesis is regulated by vascular endothelial growth factor (VEGF). Hypoxia and nutrient deprivation increase the intracellular levels of ROS by regulating VEGF expression [53, 54]. ROS levels are critical for maintaining stem cell function as well as drug resistance, which allows cancer cells to survive during treatment, resulting in both stemness and cancer-initiating capabilities [55].

In contrast to the growth promoting effect of ROS, studies suggest that the high levels of ROS induce cell cycle arrest, senescence, and apoptosis. For example, TNF receptor, a death receptor, induces ROS production through the mitochondrial electron transport chain, which leads to activation of caspases and cell death [56]. Increased oxidative stress induces senescence-mediated tumor suppression through activation of the cell-cycle inhibitor $p16^{INK4A}$ [57]. Furthermore, ROS can be cytotoxic when their levels reach a threshold that is incompatible with cellular survival, and this can inhibit cancer cell progression and thereby be therapeutic [58]. Although cancer cells express higher levels of ROS than normal cells, there are a range of levels of ROS within various cell types that are below a toxic threshold and thus compatible with cellular homeostasis. However, drug-induced ROS that cannot be neutralized by cellular antioxidant systems will exceed the toxic threshold and can therefore be used for cancer chemotherapy. This concept has recently received much attention.

5. ROS IN CANCER THERAPY

5.1. Background

Although ROS play an important role in maintaining cellular homeostasis and also in tumor development, induction of ROS in cancer cells is emerging as an important pathway that contributes to the effectiveness of many anticancer agents [23, 37, 58–60]. ROS-inducing anticancer agents enhance production of ROS that exceeds threshold levels of ROS, and this is often due to the disabling of intracellular redox pathways and cellular antioxidant capacity. There is also evidence that drug resistance in some cancer cell lines can be related to excess intracellular antioxidant capacity. Multidrug-resistant HL-60 leukemia cells express relatively high levels of catalase and are resistant to H_2O_2-induced cytotoxicity [61], and there are several examples of resistance to ROS-inducing agents, such as arsenic trioxide, taxol, and platinum derivatives, due to high levels of glutathione and redox enzymes [62–65]. For example, peroxiredoxin-3 expression is upregulated in multiple tumors including prostate cancer, and this protein catalyzes the reduction of ROS and thereby decreases cellular stress in cancer cell lines and decreases the efficacy of ROS-inducing anticancer agents [66]. Transgenic mouse models in which glutathione (GSH) and thioredoxin were depleted demonstrated that these cellular reductants were also required for tumor development [67]. Moreover, manipulation of redox levels in wild-type mice injected with cancer cell lines (e.g., MDA-MB-231 cells) showed that chemical-induced depletion of GSH or inhibition of cysteine uptake decreased tumor volume. Thus, cellular reductants play a role in tumorigenesis and are themselves potential drug targets for cancer chemotherapy, and this may be due, in part, to increased production of ROS.

5.2. Pathways for Drug-Induced ROS

The mitochondria and the mitochondrial electron transport chain are major sites for generation of intracellular ROS. Electron transport associated with complexes I and III generates free radicals, such as $O_2^{\bullet -}$ which in turn is converted to H_2O_2 by SOD. The homeostatic levels of H_2O_2 are maintained by redox enzymes, including catalase, GPx, glutathione reductase, perioxiredoxin, and thioredoxin reductase. Several ROS-inducing anticancer agents including arsenic trioxide and related arsenicals [68, 69] disrupt mitochondria to enhance $O_2^{\bullet -}$ and H_2O_2 production which cannot be handled by redox systems and therefore exceeds the upper threshold, resulting in activation of ROS-dependent cell death pathways. Arsenic trioxide binds thiol groups of enzymes involved in redox cycling, and there are reports that arsenic binds/perturbs the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane [70]. This interaction was associated with decreased mitochondrial membrane potential (MMP) and the release of mitochondrial H_2O_2 and cytochrome c with the latter response triggering the intrinsic apoptosis pathway. There is also evidence that adenine nucleotide translocase (ANT) located in the mitochondrial inner membrane is also a target of arsenicals and arsenic-induced loss of MMP and induction of apoptosis [71]. Several other structural classes of ROS-inducing anticancer agents also target mitochondria and these include some retinoids, rotenone, tanshinone 2A, gallic acid, capsaicin, jasmonales, avocatin B, the alkaloid chelerythrine, and the substituted indole, F6 [72–80]. Interestingly, there are several reports showing that the natural products, such as betulinic acid and celastrol, and synthetic oleanane triterpenoids, including CDDO-Me and related compounds, also target mitochondria and induce ROS generation [81–88]. Celastrol was a potent inhibitor of mitochondrial respiratory chain complex I in H1299 lung cancer cells [83], whereas betulinic acid induced permeabilizaton of the mitochondrial outer membrane [81, 82]. CDDO-Me-induced mitochondrial effects were cell context-dependent and appeared to enhance the permeability of the mitochondrial inner membrane [84]. These results clearly demonstrate that the mechanism of action of several anticancer agents is partially dependent on disruption of mitochondria, which are an important therapeutic target [89, 90].

The second major pathway for drug-induced ROS is due to inhibition or disabling of redox pathways or depletion of GSH [23]. Many experimental and clinically used anticancer agents act through this pathway, resulting in accumulation of cytotoxic levels of ROS in cancer cells; however, in some cases, the precise targets are not well defined. Some of these agents that have been in clinical trials include: buthionine sulfoximine and imexon, which deplete GSH levels [46, 91–95]; 2-methoxyestradiol, mangafodi-

pir, and tetrathiomolybdate, which inhibit SOD [93–95]; phenethylisothiocyanate (PEITC), which forms GSH adducts and inhibits GPx as well as complex III of the mitochondrial electron transport chain [96] and NF-κB [46]. Curcumin and curcuminoids exhibit anticancer and anti-inflammatory activities in multiple cancer cell lines, and there is continuing interest in using curcumin in clinical trials and overcoming problems associated with low bioavailability [97]. Curcumin induces ROS in some cancer cells [98, 99], and this is consistent with the irreversible inhibition of thioredoxin reductase in which curcumin alkylates residues (Cys^{496}/Sec^{497}) in the catalytic site of this enzyme [100]. Thus, ROS-inducing anticancer agents can target mitochondria and enzymes that participate in redox pathways resulting in levels of ROS that cannot be tolerated, leading to activation of genes/pathways that kill the cancer cells.

5.3. ROS-Induced Genes/Pathways

ROS-induced genes and pathways are highly variable and dependent on the specific functions of ROS which include a role in maintaining homeostasis, induction of DNA damage associated with tumor promotion and progression, and inhibition of tumor growth. Although ROS may induce common genes and pathways, such as activation of DNA damage, repair, and redox genes, ROS associated with anticancer agents activate pathways, leading to inhibition of cancer cell growth and survival. This review will focus only on a limited number of ROS-induced responses that are observed in multiple cancer cell lines and can be used for the design of combination therapies that include an ROS-inducing agent.

5.3.1. Mitochondria-Derived ROS

Mitochondria are a major source of ROS, and as indicated above, ROS-inducing agents can interact with mitochondrial membrane proteins and inhibit the mitochondrial electron transport pathways and redox enzymes to generate ROS [89, 90]. The mechanisms of drug-induced generation of ROS from mitochondria are complex and may include decreased MMP, permeabilizaton of mitochondrial membranes, modulation of the expression and levels of BH3 proteins (bcl-2, bak, bax), and release of cytochrome c, leading to activation of intrinsic apoptosis pathways [89, 90]. Generation of mitochondria-derived ROS and the resulting apoptosis contribute to the cytotoxicity of ROS-inducing anticancer agents; however, the efficacy of this class of anticancer drugs is also derived from other pathways that are briefly discussed below.

5.3.2. Changes in Gene Expression

A comprehensive time course study (1, 3, 7, and 24 hr) using H_2O_2, menadione (a quinone compound that undergoes redox cycling to give rise to $O_2^{\bullet-}$), and *t*-butyl hydroperoxide in MCF-7 breast cancer cells showed that the patterns of changes in gene expression were similar for the three ROS inducers [101]. In contrast, there were significant time-dependent differences observed for these compounds, and the three compounds modulated 421 (up- or down-regulated) genes in common over the entire time course. In addition to activation of p53 and DNA damage-regulated genes and antioxidant response genes, ROS also affected expression of genes associated with the cell cycle, signal transduction, interleukin 6, cAMP/Ca^{2+}, transcription factors, other cytokines, hormones, and protein degradation. A similar study in CaCo2 colon cells also demonstrated both similarities and overlap of genes and pathways modulated after treatment with H_2O_2 or menadione [102]. Both studies demonstrate the complexity of ROS-induced changes in gene expression but they did not focus on characterizing specific genes and pathways associated with the antineoplastic activities of ROS inducers.

O'Hagan and coworkers [103] used a different approach where they also treated SW480 colon cancer cells for 30 min and observed some distinct changes in intracellular location of some genes and increased binding of DNA methyltransferase 1 (DNMT1) and histone deacetylase sirtuin-1 (SIRT1) to chromatin. Subsequent ChIP-seq and ChIP-chip arrays demonstrated relocalization of DNMT1, SIRT1, and other proteins associated with chromatin modifying complexes. Moreover, there was a trend showing that there was a recruitment of silencing proteins to actively transcribed genes with GC-rich promoters, and this was accompanied by changes in histone marks associated with epigenetic gene repression. Thus, genes such as c-Myc and ACTB (with GC-rich promoters) were decreased by H_2O_2, whereas most high expression genes that do not have GC-rich promoters were either increased or unchanged [103]. This study

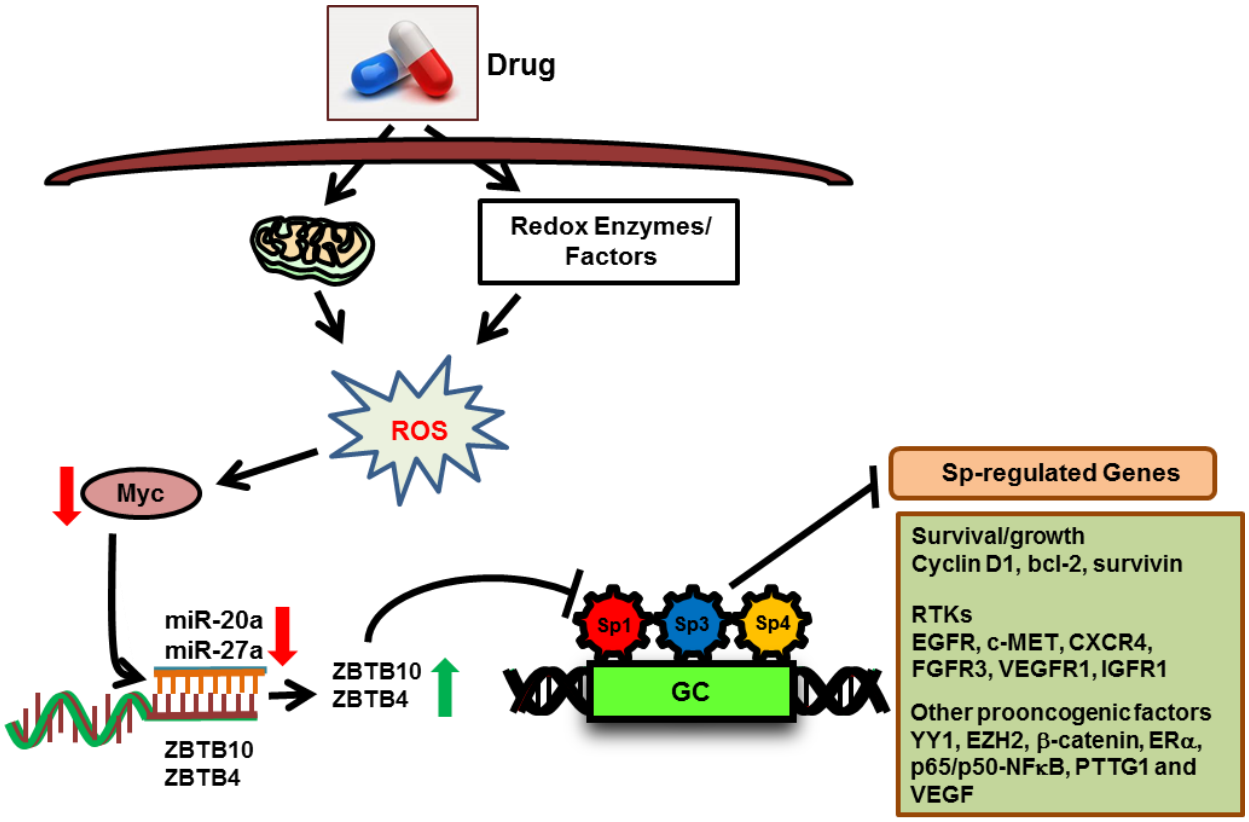

FIGURE 1. ROS-inducing anticancer agents downregulate Sp transcription factors. ROS-inducing anticancer agents induce a cascade of events in which ROS-dependent epigenetic downregulation of c-Myc causes decreased expression of c-Myc-regulated miR-27a and miR-20a/miR-17-5p, resulting in the induction of miR-regulated transcriptional repressors ZBTB10/ZBTB34 and ZBTB4, respectively. The ZBTB repressors bind GC-rich sites to displace Sp1, Sp3, and Sp4 [119–127].

demonstrated that some of the initial rapid changes induced by H_2O_2 in transcriptional silencing were due, in part, to epigenetic pathways, and these results provided a key insight on a hitherto unknown ROS-inducing antineoplastic pathway.

5.3.3. Downregulation of Sp Transcription Factors and Pro-oncogenic Sp-Regulated Genes

Sp1, Sp3, and Sp4 proteins are highly expressed in cancer cells and tumors, and high Sp1 expression in tumors is a negative prognostic indicator for lung, pancreatic, gastric, glioma, prostate, and breast cancer patient survival [104–118]. Results of knockdown studies in cancer cell lines demonstrate that Sp transcription factors play a role in cancer cell proliferation, survival, and migration/invasion (**Figure 1**). Initial studies showed that CDDO-Me, betulinic acid, a nitro-aspirin derivative, curcumin, and celastrol decreased expression of Sp1, Sp3, and Sp4 as well as pro-oncogenic Sp-regulated genes in several cancer cell lines [98, 119–123]. Moreover, the effects of the ROS-inducing agents on cell proliferation and Sp downregulation were attenuated after cotreatment with the antioxidant GSH. In addition, H_2O_2, ascorbate, and *t*-butyl hydroperoxide also decreased expression of Sp1, Sp3, and Sp4 [119–121]. In parallel studies, research in our laboratory reported that high expression of Sp transcription factors in many cancer cell lines was due to microRNA-27a (miR-27a)-

dependent suppression of ZBTB10, an Sp transcriptional repressor that competitively binds GC-rich sites to displace Sp proteins [124]. Since Sp1, Sp3, and Sp4 have GC-rich Sp promoters, ZBTB10 can directly downregulate all three transcription factors. Subsequent studies showed that miR-20a and miR-17-5p suppressed ZBTB4 [125], and miR-27a suppressed ZBTB34 in cancer cell lines, and overexpression of the ZBTB genes or treatment of cells with miR antagomirs decreased expression of Sp1, Sp3, Sp4, and Sp-regulated genes [124–127]. Moreover, ROS-inducing agents decreased miR-27a, miR-20a, and miR-17-5p, and induced ZBTB10, ZBTB4, and ZBTB34, and these responses were also attenuated after cotreatment with GSH confirming that ROS-induced downregulation of Sp1, Sp3, and Sp4 is due to ROS-dependent disruption of miR-ZBTB interactions. The key missing step in triggering the ROS–Sp (downregulation) cascade was the mechanism of downregulation of miR-27a/miR-20a/miR-17-5p. There was evidence that the miR-23a~24a~24-2 and miR-17~92 clusters that encode miR-27a and miR-20a/miR-17-5p, respectively, were regulated by c-Myc [128–130]. Two recent studies using PEITC and histone deacetylase inhibitors in pancreatic and rhabdomyosarcoma cells, respectively, show that both compounds rapidly induce ROS and downregulate c-Myc (within 3 hr) [126, 127]. Moreover, the rapid decrease in c-Myc was accompanied by changes in histone methylation and/or acetylation [126, 127], and these results were consistent with H_2O_2-induced repression of c-Myc through recruitment of chromatin modifying complexes to the c-Myc promoter [103]. Similar results were observed for Sp1 downregulation in both pancreatic cancer and rhabdomyosarcoma cells, and knockdown of c-Myc by RNA interference mimicked the effects of ROS. These results demonstrate an important ROS-induced pathway in cancer cells, resulting in downregulation of Sp-regulated genes that are important for cell proliferation, survival, and migration/invasion [131].

5.3.4. Potentiation of Activation of p38 and c-Jun N-Terminal Kinase (JNK)

Stress kinases such as p38 and JNK are sensitive to some ROS-inducing drugs, and one report showed that cisplatin-dependent induction of these stress kinases was ROS-dependent and inhibited by antioxidants [132]. Similar results were observed in lung cancer cells treated with celastrol, and both an ROS inhibitor (*N*-acetylcysteine) and a JNK inhibitor (SP600125) suppressed cell death and cell death pathways [83]. There is also evidence that apoptosis signaling regulated kinase 1 (ASK-1), which is upstream from p38/JNK, may be an ROS target, which in turn activates stress kinase-dependent apoptosis pathway [133-135].

5.3.5. Inhibition of mTOR and Induction of Autophagy

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is widely expressed. mTOR is a key regulatory kinase that plays a role in regulating ribosomal translation of mRNA into proteins and is important for cell growth, survival, and autophagy [136]. mTOR signaling is amplified in many cancers, and the search for effective inhibitors of mTOR is a major effort of many pharmaceutical companies [137]. mTOR is activated by nutrients, growth factors, and other stimuli and integrates signals from multiple upstream kinases such as the PI3K-Akt and Ras-Raf kinase pathways, and inhibitors of these pathways, such as PTEN (phosphatase and tensin homolog, a natural inhibitor of PI3K-Akt), also inhibit mTOR. AMP-activated protein kinase (AMPK) is also a prominent upstream regulator, and activation of AMPK by liver kinase B1 (LKB1) or sestrin 2 results in phosphorylation of the tuberous sclerosis complex (TSC) which also inhibits mTOR through inhibiting ras homolog enriched in brain (RHEB). This complex pathway and its key components vary among tumor types, and chemotherapies that target mTOR include both direct inhibitors, such as everolimus and temsirolimus, and also upstream kinase inhibitors [137, 138]. ROS directly activate AMPKα through S-glutathionylation of cysteine 299 of AMPKα, and AMPKα inhibits mTOR [139, 140]. ROS-dependent activation of p53 results in the induction of two p53-regulated genes, namely, sestrin 1 and sestrin 2, which inhibit mTOR signaling by activation of AMPKα [141–143]. This ROS-induced pathway can be both p53-dependent and p53-independent and thereby represents a viable therapeutic option for inhibition of mTOR signaling in various tumors. In addition, ROS also induced autophagy which can be either a protective or a cytotoxic response [144, 145]. The effects of different

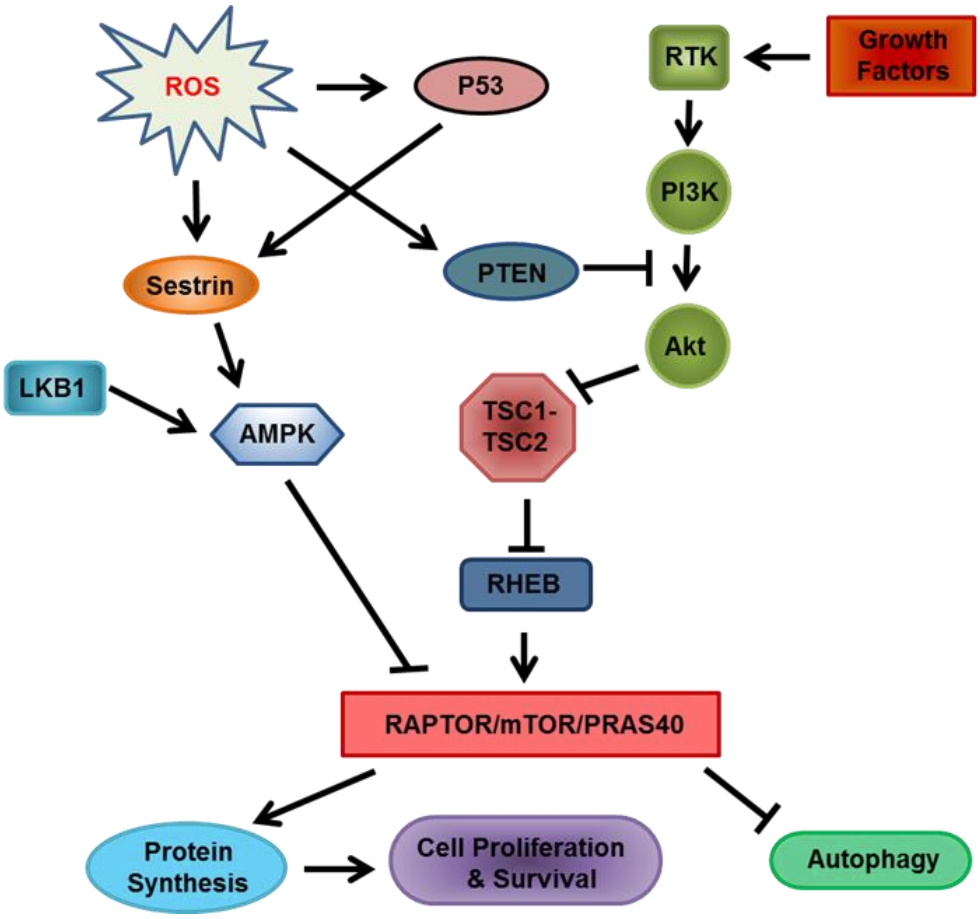

FIGURE 2. Multiple pathways for ROS-dependent inhibition of mTOR. mTOR inhibitors can directly block mTOR signaling; however, mTOR inhibition can be achieved by targeting upstream factors such as Akt (inhibition) or sestrin (activation), which in turn modulate TSC1/TSC2 or AMPKα activity, respectively.

ROS inducers are cell context dependent; for example, some agents such as curcumin induce autophagic cell death in colon cancer cells [146], and presumably the ROS-dependent inhibition of mTOR by curcumin also contributes to this response (**Figure 2**).

In summary, ROS-inducing anticancer agents represent an important and underutilized approach for cancer chemotherapy and these drugs can be specifically targeted for tumors that already express high ROS levels. Moreover, ROS inducers can also be effective for combined therapies since many Sp-regulated genes (**Figure 1**) also play roles in drug- and radiation-resistance.

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