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Mini-review

Harnessing altered oxidative metabolism in cancer by augmented prooxidant therapy

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ABSTRACT

Deregulated metabolism of oxygen with increased generation of reactive oxygen species (ROS) is characteristic for a majority of cancers. The elevated ROS levels are in part responsible for further progression of cancer, but when produced in large excess, they endanger the viability of the cancer cells. To protect themselves from ROS-mediated toxicity, many types of cancers enhance the intrinsic antioxidant defenses, which make them dependent on the efficacy of a given ROS-detoxifying system. This poses an attractive target for anticancer therapy by two main approaches: the use of ROS-generating agents (i.e., prooxidants) or by inhibition of a chosen antioxidant system. However, the clinical efficacy of either of these approaches used alone is modest at best. The solution may rely on combining these strategies into an advanced prooxidant therapy (APoT) in order to produce a synergistic and cancer-specific effect. Indeed, such strategies have proven efficient in preclinical models, e.g., in B cell malignancies and breast cancer. Following promising experimental reports on APoT, this approach needs to be further extensively tested in order to become a potential alternative or an enhancement for classical chemotherapy.

1. Introduction

Oxygen is necessary for the survival of all aerobic organisms on Earth, and its utilization in biochemical reactions is one of the bases for cellular bioenergetics. Also, in humans, metabolites of oxygen are integral components of multiple cellular processes. And yet, when oxygen metabolism produces excessive, poorly controlled amounts of reactive oxygen species (ROS), it becomes harmful to the cell. The excess of ROS generation leads to deleterious effects on the cell by affecting the chemical structure and/or function of DNA, proteins, lipids, and other macromolecules. ROS can also be supplied to the cells externally, from diet or the environment in general. Therefore, most living organisms have developed several tightly regulated mechanisms for scavenging these reactive species. Pathological disruption of these mechanisms can elicit the same deleterious effects on cells as the overproduction of ROS. The excessive imbalance between the production or intake of ROS and the effectiveness of particular antioxidant systems is often referred to as “oxidative stress”. Accumulating evidence indicates the engagement of sustained oxidative stress in the development and progression of multiple diseases, including cancer. At the same time, the exaggerated oxidative stress in tumor cells constitutes a potential target for anticancer therapies. Two main anticancer approaches interfering with oxidative metabolism are being considered: the use of antioxidants, which might be of use in cancer prevention, but may paradoxically produce adverse effects in advanced cancer, and the prooxidant treatment, which is the subject of the current review.

2. Sources of ROS

Sources of ROS can be divided into exogenous and endogenous. Exogenous ROS come mainly from pollutants, smoke, xenobiotics, but also some drugs or ionizing radiation and are responsible in part for the carcinogenic effects of these factors. Endogenous ROS are constantly produced in various metabolic pathways in mitochondria, peroxisomes, endoplasmic reticulum, and in cell membranes [1]. Mitochondrial electron transport chain (ETC) is the major producer of superoxide radicals ($O_2^{\bullet -}$) in most mammalian tissues [2–4]. The second main contributors are the NADPH oxidase (NOX) family enzymes, namely

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Abbreviations

AhR	aryl hydrocarbon receptor
AML	acute myeloid leukemia
AP-1	activator protein 1
APoT	augmented prooxidant therapy
ARE	antioxidant-responsive element
ASC	ascorbate
AREG	amphiregulin
ATO	arsenic trioxide
AUR	auranofin
BCP-ALL	B cell precursor acute lymphoblastic leukemia
BSO	buthionine sulfoximine
CAT	catalase
CLL	chronic lymphocytic leukemia
CRISPR	clustered regularly interspaced short palindromic repeats
DHA	dehydroascorbic acid
DLBCL	diffuse large B-cell lymphoma
DUB	deubiquitinase
DUOX	dual oxidase
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchymal transition
ETC	electron transport chain
GCL	glutamate-cysteine ligase
γ-GCS	γ-glutamylcysteine synthetase
GLUT	glucose transporter
GOx	glucose oxidase
GPx	glutathione peroxidase
GSH	glutathione
GSS	glutathione synthetase
GSSG	disulfide-oxidized glutathione
GSR	glutathione reductase
GST	glutathione S-transferase
HMOX1	heme oxygenase 1
HIF-1α	hypoxia inducible transcription factor-1α
IDH	isocytrate dehydrogenase
KEAP-1	Kelch-like ECH associated protein 1
MAPK	mitogen-activated protein kinase
MEN	menadione
MDSC	myeloid-derived suppressor cell
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF_kB	nuclear factor $_k$B
NOX	NADPH oxidase
NRF2	nuclear factor erythroid 2-related factor 2
OXPHOS	oxidative phosphorylation
PD-L1	programmed death ligand 1
PPP	pentose phosphate pathway
PRDX	peroxiredoxin
PTGR	prostaglandin reductase
ROS	reactive oxygen species
SCF	stem cell factor
SOD	superoxide dismutase
TAM	tumor-associated macrophage
TET	ten eleven translocation
TME	tumor microenvironment
TNBC	triple-negative breast cancer
TNF	tumor necrosis factor
Treg	regulatory T cell
TPRA1	transient receptor potential cation channel, subfamily A, member 1
TXN	thioredoxin
TXNRD	thioredoxin reductase
xc	cysteine transporter

NOX1-5, dual oxidase 1 (DUOX1), and dual oxidase 2 (DUOX2) that have the sole function of generating ROS instead of generating ROS as a byproduct. When activated, NOX1-3 mainly generate $O_2^{\bullet -}$, while NOX4, DUOX1, and DUOX2 are capable of producing hydrogen peroxide (H_2O_2) directly [5]. Other important endogenous sources of ROS are enzymes such as xanthine oxidase [6], lipoxygenases [7], and cytochrome P450 [8].

3. Role of ROS in cancer initiation and progression

Numerous reports have described in detail the role of increased ROS concentrations in malignant transformation and the impact of oxidative stress on the antioxidant adaptation of cancer cells [9–11]. Briefly, moderate ROS levels, and especially H_2O_2, increase intracellular pro-proliferative signaling from the growth receptors, which augments uncontrolled cell growth. This growth, in turn, requires the additional fuel to cover the metabolic demand of the cells, which in most of the cases cannot be fulfilled and results in glucose deprivation and an even greater increase of the oxidative stress [12]. Additionally, persistent oxidative stress directly triggers DNA damage and subsequently results in the induction of some oncogenic mutations that increase cellular metabolism and trigger even more elevated ROS production [13,14]. To counterbalance the oxidative stress, cancer cells activate redox-responsive transcription factors such as the nuclear factor erythroid 2-related factor 2 (NRF2), activator protein 1 (AP-1), nuclear factor $_k$B (NF_kB), hypoxia inducible transcription factor 1a (HIF-1a), and p53 [15–18]. This subsequently leads to an increase in the levels of the various components of antioxidant systems that are directly engaged in maintaining the viability of cancer cells in a highly oxidative milieu [11]. Recently, also a novel anti-apoptotic pathway that relies on TRPA1, a neuronal redox-sensing Ca^{2+}-influx channel, was discovered. TRPA1 does not affect canonical ROS-neutralizing programs relying on antioxidants but senses redox stress and upregulates Ca^{2+}-dependent anti-apoptotic programs that promote oxidative-stress tolerance [19].

Another layer of complexity is added by the interplay among ROS, cancer cells, and tumor microenvironment (TME). It was shown, for instance, that triple-negative breast cancer (TNBC) exhibits a high overall level of ROS and simultaneously high dependency on ROS for survival [20]. Elevated ROS levels in the TNBC subtype, basal-like/BRCA1, trigger aryl hydrocarbon receptor (AhR) nuclear accumulation and activation to promote the transcription of both, antioxidant enzymes and the epidermal growth factor receptor (EGFR) ligand, amphiregulin (AREG). As observed, this activation in a mouse model of BRCA1-related basal breast cancer led to a potent production of chemokines that recruited monocytes and activated the proangiogenic function of macrophages in the tumor microenvironment. Moreover, CRISPR-mediated knockout of either of the genes – AhR or AREG in the human basal-like breast cancer cell lines resulted in their impaired growth and further sensitization to EGFR inhibitors suggesting that tailored inhibition of AhR-regulated pathways can lead to breast cancer eradication by pushing it beyond its ROS tolerance limit and depriving it of tumor-supporting immune cells [21]. In a mouse model of TNBC, ROS triggered either by chemotherapy (paclitaxel) or antioxidant depletion (BSO), induced NF-κB signaling-dependent expression of programmed death ligand 1 (PD-L1) in macrophages residing in TME. Enhancing paclitaxel therapy with an anti-mouse PD-L1 blocking antibody significantly reduced tumor burden and increased the number of tumor-associated cytotoxic T cells [22]. An alternative approach was proposed by Raninga et al. that adapted auranofin (AUR) for the treatment of TNBC and observed pERK1/2-dependent up-regulation of PD-L1 on the surface of cancer cells. Indeed, in the syngeneic 4T1.2 cell line model, AUR treatment in combination with the PD-L1 blocking

antibody synergistically inhibited the growth of tumors [23]. It was also shown that a tumor might promote metabolically-adapted, suppressive neutrophils. In a murine model of breast cancer (4T1 cells), aberrant stem cell factor (SCF)/c-Kit signaling increased oxidative phosphorylation and NADPH-oxidase activity in neutrophils, which resulted in the elevation of ROS levels and local immunosuppression [24].

All in all, oxidative stress elicits variable effects on subsequent stages of cancer development and those effects are related to the antioxidant adaption of cancer cells to elevated ROS levels. The contribution of ROS in cancer development and progression is presented schematically in Fig. 1.

4. Antioxidant defense systems in cancer

To prevent the deleterious effects of ROS, cells have developed numerous parallel mechanisms for their scavenging, including endogenous antioxidant enzymes (e.g., superoxide dismutases, catalase, glutathione peroxidases, thioredoxins/thioredoxin reductases, and peroxiredoxins), and non-enzymatic antioxidant molecules (e.g., glutathione, coenzyme Q, ferritin, and bilirubin). Interestingly, in advanced stages of cancer, the intrinsic antioxidants are currently seen as protectors of cancer cells and constitute a tempting therapeutic target, as elaborated below.

4.1. NRF2, a critical transcription factor that maintains redox homeostasis

The complex network of antioxidant systems is often under the fine-tuning control of the NRF2 signaling pathway [25–27]. Under normal conditions, NRF2 is negatively regulated by Kelch-like ECH associated protein 1 (KEAP-1), a protein adaptor for RING E3 ubiquitin ligase, which promotes degradation of NRF2 by the proteasome. However, as KEAP-1 is a cysteine-rich intracellular oxidative sensor, under conditions of oxidative stress, its thiol groups are oxidized, which promotes NRF2 dissociation, stabilization, and translocation to the nucleus [28]. There, upon binding to the antioxidant-responsive elements (ARE), NRF2 initiates transcription of various cytoprotective enzymes such as heme oxygenase 1 (HMOX1), glutamate-cysteine ligase (GCL), peroxiredoxin 1 (PRDX1) and glutathione S-transferases (GST) [29,30]. Hence, NRF2 is regarded as a transcription factor that protects normal cells from excessive cellular damage evoked by metabolic, xenobiotic, and oxidative stress, and the associated carcinogenesis, and therefore, it has been traditionally deemed as a tumor suppressor [25,31]. Conversely, in cancer cells NRF2 is frequently upregulated, and protects the cells from the consequences of excessive oxidative stress, chemotherapeutic agents, or radiotherapy, indicating NRF2 as a promising target for anticancer therapies [32].

4.2. Superoxide dismutase

Superoxide dismutase (SOD) is one of the most potent intracellular enzymatic antioxidants responsible for the conversion of two $O_2^{\bullet-}$ anions into molecular oxygen and H_2O_2. There are three isoforms of SOD identified in mammals: zinc-copper SOD1 located mainly in the cytoplasm, mitochondrial manganese SOD2, and extracellular SOD3 [33]. Among them, the SOD1 has been confirmed to play a crucial cytoprotective role, as SOD1 knock-out mice die shortly after birth [34]. Still, due to the seemingly more pronounced role of SOD1 in cancer than in normal cells, targeting this molecule is considered as a putative anticancer approach [35].

4.3. Catalase

Catalase (CAT) is a heme-dependent enzyme mainly located in peroxisomes. The regulation of catalase expression appears to be mainly controlled at the transcriptional level, although other mechanisms may also be involved [36]. The role of CAT in tumorigenesis is unclear. Both, increase and decrease in CAT expression have been reported in cancer tissues as compared to their normal counterparts, and opposing results

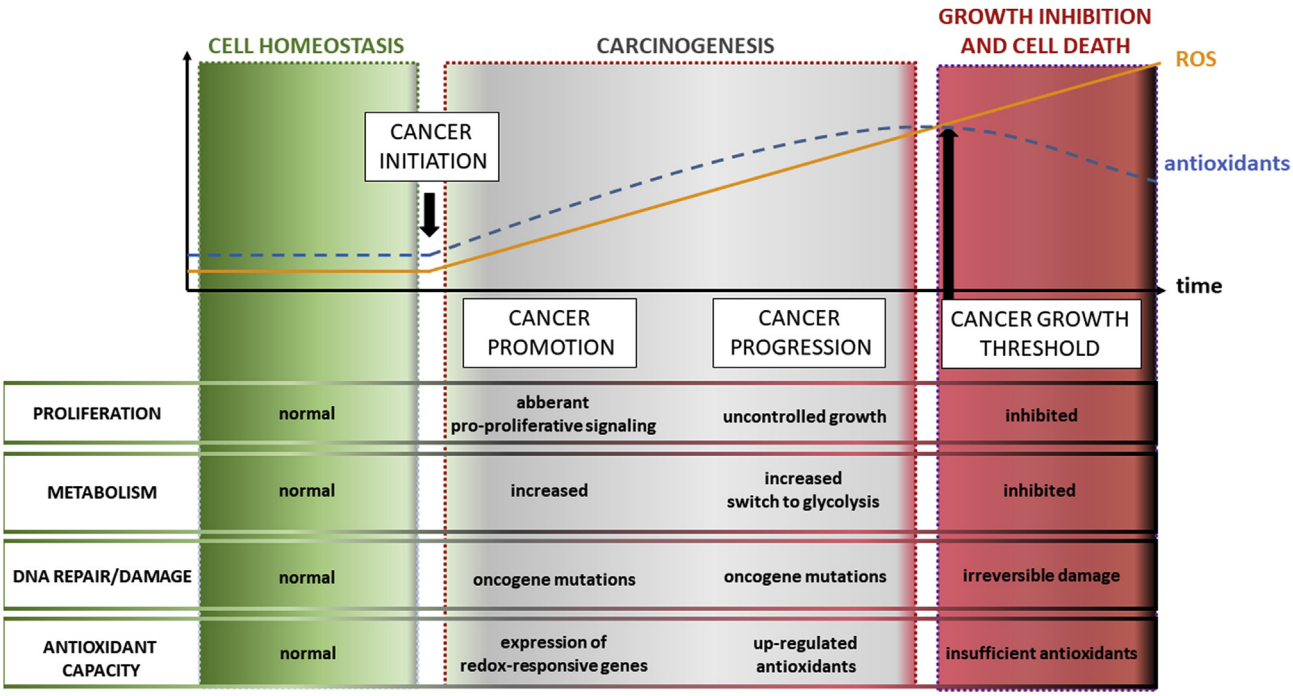

Fig. 1. Schematic representation of ROS contribution to cancer development. Normal cells are characterized by cell homeostasis and low level of ROS. Carcinogenesis is accompanied by the gradual increase of ROS levels that influence several layers of cell biology. Increased ROS levels stimulate cell proliferation and, in turn, fuel up metabolism. Exaggerated oxidative stress triggers DNA damage and, consequently, mutational burden, but also stimulate the expression of genes involved in antioxidant machinery and overall antioxidant capacity. When oxidative stress exceeds the adaptive threshold of antioxidant capacity of cancer cells, it causes irreversible damage to cellular macromolecules that, eventually, leads to cancer cell death.

have been obtained regarding its potential role in cancer cell resistance against chemotherapeutic agents [37–39]. That suggests the role of CAT in tumors may be cancer type-dependent. Nevertheless, there is an agreement that CAT-overexpressing cells are more resistant to prooxidant intervention [39,40].

4.4. GSH and GSH-related enzymes

Glutathione (GSH) is a short peptide (γ-L-glutamyl-L-cysteinyl-glycine) that reaches millimolar (0.5–10 mM) concentrations within the cells and has multiple roles in the regulation of cellular homeostasis [41]. *De novo* synthesis of GSH is catalyzed by two enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GSS), while glutathione reductase (GSR) is involved in the recycling of oxidized glutathione using NADPH [1,42]. GSH, together with its disulfide-oxidized (GSSG) form, serve as a major redox couple in animal cells. A decrease in the ratio between GSH and GSSG leads to an increased susceptibility to oxidative stress and carcinogenesis, while the elevated GSH levels increase the antioxidant capacity of many cancer cells, enhancing their resistance to oxidative stress [43].

Glutathione peroxidases (GPx) are enzymes that rely on GSH reducing capacity. GPx catalyze the reduction of H_2O_2 and other peroxides at the expense of oxidizing GSH to GSSG [44]. In mammals, so far, the eight isoforms of GPxs have been identified and can be classified into two groups: selenium-containing GPxs (GPx1-4 and 6), as well as their non-selenium congeners (GPx5, 7 and 8). Changes in GPxs levels have been reported in several types of cancers [45–47], but their usefulness as potential targets in cancers is still not clear [44,48].

4.5. Thioredoxin system and peroxiredoxins

Thioredoxin (TXN) system maintains the redox balance by mutually dependent antioxidant enzymes such as thioredoxins (TXNs) and thioredoxin reductases (TXNRDs) [49]. In mammals, both TXNs and TXNRDs are predominantly expressed as cytosolic (TXN1, TXNRD1) and mitochondrial (TXN2, TXNRD2) isoforms [50]. All four enzymes are essential, and their deficiency causes early embryonic lethality in mice [51,52]. TXNs play multiple functions in cells and regulate cell growth, proliferation, chemotaxis, and immune response [53]. By means of their active site cysteine thiols, TXNs reduce disulfides of other proteins, including other enzymes (apoptosis signaling kinase-1, ribonucleotide reductase, peroxiredoxins), as well as transcription factors (AP-1, NF-κB, HIF1a) [54]. During catalysis, cysteines in the active site of TXN form a disulfide, which is subsequently reduced by NADPH-dependent TXNRDs [55].

In cancer cells, a dysregulation of the TXN system has been reported. For instance, in diffuse large B-cell lymphoma (DLBCL) patients, increased expression of TXN1 correlated with poor prognosis [56]. Moreover, increased expression of TXN system-associated antioxidant enzymes was found in human colorectal [57], hepatocellular [58], and breast [59] cancer cells, as well as in Burkitt lymphoma [60]. These reports suggest that increased expression of the TXN-related enzymes promotes the survival of cancer cells under oxidative stress conditions.

As mentioned above, the TXN system reduces and reactivates enzymes fundamental for H_2O_2 and other peroxides' removal – PRDXs [61]. Besides their major antioxidant function, PRDXs play a role in multiple signaling cascades and cellular functions via direct interactions with other proteins [62–65]. PRDXs are present at higher concentrations in the cell than their major reductant - TXN, which results in the accumulation of oxidized forms of PRDXs, which then oligomerize and function as cellular chaperones [66]. In mammals, there are six isoforms of PRDXs located in various subcellular compartments. PRDX1 was suggested to function as a tumor suppressor since PRDX1 knock-out mice die prematurely, among others, from cancer [67]. Although the role of PRDX1 has been described in many tumors, its significance for the prognosis and tumor development is multifaceted [68]. Generally, however, recent data suggest that PRDX1 is one of the gatekeepers of cancer cell survival [69]. It has also been reported that PRDX1 and PRDX2 may serve as biomarkers or targets for anticancer therapies in different types of malignancies [60,70,71].

4.6. Crosstalk between antioxidant defense systems

In both, physiological and pathological conditions, some antioxidants function in a correlated manner and a change in the activity of one of them can be compensated by the respective changes in the activities of the others. For instance, it has been shown that cancers with disturbed GSH homeostasis may be targeted by a synthetic lethality strategy with TXNRD inhibitors [72]. Similarly, it has been reported that concomitant inhibition of GSH and TXN systems induces a synergistic cancer cell death *in vitro* and *in vivo*, indicating the supplementary role of these two antioxidants in cancer [59].

5. Inhibitors of antioxidant systems as anticancer drugs

Since overexpression of enzymes of particular antioxidant systems has been observed in cancer, inhibition of these pathways became an interesting anticancer approach. Hereby, we will focus mainly on clinically available, direct inhibitors of antioxidant systems with anticancer activity, including those tested based on drugs' repurposing approach.

5.1. Inhibitors of GSH system

The decrease of GSH is a robust ROS-inducing anticancer approach, which may be achieved either by blocking of the GSH synthesis or by its depletion. The former approach is possible at different stages of the GSH synthesis pathway. For instance, buthionine sulfoximine (BSO), a compound tested in clinical trials in combination with melphalan against neuroblastoma [73], blocks GCL, and thus interferes with the first step of GSH synthesis. Sulfasalazine, on the other hand, inhibits cysteine transporter (xc-) and thus decreases GSH pool. Sulfasalazine is clinically approved for the treatment of rheumatoid arthritis and currently studied against various cancers, both in preclinical [74,75] and clinical trials (NCT03847311). The second approach, which involves GSH depletion, may be exemplified by APR-246 (PRIMA-1^{Met}), which forms covalent adducts with GSH, disturbs GSH intracellular recycling, and induces ROS [76]. APR-246 is mostly recognized as the p53 reactivating drug, and its efficacy in combination with azacitidine is tested in an ongoing phase III clinical trial in patients with p53-mutated myelodysplastic syndromes (NCT03745716). Liu et al. [77] demonstrated that APR-246-mediated decrease of GSH accounts for its anticancer effects, mainly through ROS-induced lipid peroxidation. Importantly, this effect was further improved by the addition of xc-transporter inhibitors, which was detected both *in vitro* and *in vivo*.

5.2. Inhibitors of TXN system

Many drugs targeting the enzymes of the TXN system have been identified and classified either as natural or synthetic compounds. These agents have been already summarized in a number of comprehensive reviews [78,79]. In this review, we will focus on the selected, clinically most promising agents.

Among natural molecules with anticancer properties that have been shown to block TXN system are curcumin and its analogs [80], piperlongumine [81], arsenic trioxide [82], and adenanthin [83]. All these compounds inhibit TXNRD, the superordinate enzyme of the TXN system; however, they are not selective. Following the raising interest of curcumin as anticancer agent currently tested in many pre-clinical and clinical trials, many analogs were designed to improve its limited bioavailability. Indeed, two curcumin derivatives, B19 and B63 were presented as potent TXNRD inhibitors with anti-tumor activity against

gastric cancer *in vitro* and *in vivo* [80,84]. Both drugs increased cellular ROS levels; however, B63 induced paraptosis-like cell death, as compared to classical caspase-dependent apoptosis mediated by B19. Piperlongumine, initially described as an inhibitor of glutathione S-transferase P (GSTP1), was recently shown to block TXNRD1 with even higher affinity [81]. Piperlongumine provoked cell death through TXNRD1 inhibition and subsequent ROS upregulation in gastric carcinoma, and also displayed radiosensitizing effects in colorectal cancer [85]. Adenanthin was shown to block many elements of the TXN system, including PRDX1, PRDX2, TXN1 [86], and TXNRD1 [83]. In line with these observations, our team has recently discovered that adenanthin effectively kills B cell precursor acute lymphoblastic leukemia cells (BCP-ALL) *in vitro* [87].

AUR represents a group of synthetic TXN system inhibitors containing metal complexes. This organic gold agent is approved by FDA for the treatment of rheumatoid arthritis since 1985 and is being tested in many pre-clinical studies and clinical trials against various types of cancer, including chronic lymphocytic leukemia (NCT01419691), small and non-small cell lung cancer (NCT01737502), or ovarian cancer (NCT03456700). We found that AUR could be repurposed as a potent anti-leukemic agent, as proved *in vitro* and *in vivo* employing a high-risk xenograft model of BCP-ALL [87]. In addition to already known AUR targets, such as cytosolic TXNRD1 and mitochondrial TXNRD2 [88], it was also shown to affect proteasome-degradation pathway by inhibition of deubiquitinases (DUBs) [89]. Nevertheless, a recent paper showed that AUR indeed inhibits DUBs, but at much higher concentrations, thus claiming TXNRD1/2 as its main targets [90]. Recently, it was presented in breast cancer cell lines that, in parallel to TXNRD1/2, AUR also depletes GSH [91].

5.3. Towards more specific antioxidant system inhibitors – limitations and perspectives

In spite of the well documented potential of targeting ROS defense mechanisms in cancer, there are practically no clinically approved drugs with strict preference towards a particular antioxidant enzyme. Lack of selectivity is mostly caused by the fact that many commonly used compounds react with nucleophilic Cys residues, which are present in active sites of many enzymes. Consequently, finding more selective inhibitors is challenging. Recently, Stafford et al. [92] described Tri-1, the selective inhibitor of cytosolic TXNRD1, using a high-throughput screening approach. The anticancer activity of this drug was evaluated *in vivo* employing syngeneic and human xenograft models of breast cancer. In both cases, Tri-1 attenuated the growth of the tumors and exerted similar anticancer effect to pan-TXNRD inhibitor, AUR. Nonetheless, due to its more selective profile, it exerted reduced toxicity to normal cells, thus showing that specific antioxidant enzymes inhibitors may possess increased potential in clinical applications. Furthermore, Zhang et al. proposed other possible approaches that could improve the selectivity of TXN-directed inhibitors, including targeting of the selenol group, inhibiting the interaction between TXN and TXNRD or TXN system-activated pro-drugs [78].

6. Prooxidants

Prooxidants may be broadly defined as agents capable of significantly elevating ROS levels. The efficacy of many well-recognized anticancer therapeutic modalities such as radiotherapy, chemotherapy, and photodynamic therapy is associated with ROS induction. They trigger ROS generation in cells through a variety of mechanisms, which are extensively summarized in Refs. [93–95]. Interestingly, selected antioxidant systems' inhibitors such as curcumin, piperlongumine, AUR, Tri-1, covalently bind to TXNRD and convert it from an antioxidant to a ROS-generating enzyme. All the above compounds have strong antitumor activity, resulting from their prooxidant properties [92,96,97]. Hereby, we focus mainly on a particular group of prooxidants, namely on those agents which are able to generate ROS extracellularly, either *in vitro* in culture media or *in vivo* in extracellular fluids. Paradoxically, chemicals that at physiological concentrations act as antioxidants, at certain conditions (high concentrations, in the presence of transition metal ions) can generate significant amounts of ROS. Examples include tocopherol [98], N-acetylcysteine and other thiols [99], polyphenols [100], carotenoids [101], fatty acids [102], indoles [103], menadione [104], and L-ascorbate (L-ASC) [105]. Many of these compounds exert antitumor activity in various preclinical cancer models. Hereby, we focus on L-ASC, which has recently returned to the spotlight due to its anticancer activity associated with its prooxidant properties, which were observed in preclinical and also in clinical studies.

6.1. Ascorbate (ASC) – chemical and biological properties

ASC has two chiral centers; therefore, there are four ASC stereoisomers. All isomers may serve as antioxidants. However, only one stereoisomer, L-ASC, has antiscorbutic activity and is recognized as vitamin C. Most organisms are able to synthesize L-ASC, but humans lack the key L-ASC biosynthesis enzyme, hence for humans, L-ASC is an essential nutrient which must be supplemented with diet [106]. In physiological concentrations, reduced L-ASC has antioxidant properties. In a culture medium, in the presence of oxidizing agents, L-ASC is oxidized first to an ascorbate radical, and then with a loss of a second electron, to L-dehydroascorbic acid (L-DHA). L-ASC and L-DHA enter cells using distinct transporter receptors [107]. Following cell entry, L-DHA is reduced back to L-ASC at the expense of the GSH and TXN systems [108].

6.2. L-ASC as an anticancer agent

Many *in vitro* studies revealed that at high concentrations (1–100 mM) L-ASC is selectively toxic to a variety of cultured tumor cell lines [105]. Importantly, preclinical *in vivo* studies in animal models revealed that high doses (4–6.5 g/kg body weight) of L-ASC given parenterally resulted in millimolar plasma concentrations, were safe, and had antitumor activity in several xenograft as well as in syngeneic murine models [109,110].

The beneficial effects of intravenous administration of high doses (10 g per day) of L-ASC to cancer patients were noted already in the mid-20th century. The following studies by Cameron and Pauling [111,112] conducted on a group of patients with advanced cancers revealed over 4-fold increase in mean survival time for L-ASC-treated subjects. In contrast, subsequent randomized controlled clinical trials [113] reported no benefit of oral L-ASC (10 g daily) to cancer patients. However, later pharmacokinetic studies revealed that intravenous L-ASC results in about 1–20 mM plasma concentrations, which are about 50-fold higher compared to orally administered L-ASC, and comparable to those that are cytotoxic to cancer cell lines *in vitro* [114]. These results led to the recognition of a critical role of the route of L-ASC delivery and triggered further clinical trials with L-ASC given parenterally, in high (10–100 g) daily doses. Thus far, there is no convincing evidence from high-quality, large-scale, randomized control clinical trials that L-ASC per se has robust antitumor effects. However, doses as high as 1.5 g/kg per day are safe and well tolerated by cancer patients, except for those with renal dysfunction and deficiency of glucose-6-phosphate dehydrogenase [115,116]. It was shown that L-ASC mitigates the side effects of conventional therapy in breast [117], pancreatic [118], and ovarian [119] cancer patients. Importantly, recent reports from early stage clinical trials evaluating the benefit of L-ASC addition to chemo- or radiotherapy are very promising [120,121]. Clinical trials evaluating the efficacy of parenteral high-dose L-ASC in combination with conventional therapy in lung, prostate, pancreatic, ovarian, and other cancers are ongoing. Recent progress and the perspectives of the anticancer utility of pharmacological L-ASC are

discussed in a recent review [122].

6.3. Mechanisms of L-ASC antitumor activity

Both pharmacologic (i.e., given parenterally, in high doses) and physiologic (achieved by oral dosing, up to 50 μM) L-ASC have antitumor properties; however, their mechanisms differ. The antitumor activity of pharmacologic L-ASC is associated with its prooxidant properties. Chen et al. [105] presented that in millimolar concentrations, and in the presence of transition metal ions present in serum, L-ASC extracellularly generates H_2O_2 in amounts that are selectively toxic to tumor cells. Another study by Yun et al. [123] reported distinct mechanisms of L-ASC antitumor action, associated with L-ASC transport to cells and intracellular generation of ROS. The authors presented that L-ASC is selectively toxic to cancer cells with B-Raf and K-Ras mutations, which activate mitogen-activated protein kinases (MAPK) pathway stimulating expression of GLUT-1, a transporter of L-DHA.

In contrast, L-ASC at physiologic concentrations exerts its antitumor effects by modulation of epigenetic pathways. As a co-factor of ten-eleven translocation (TET) enzymes, it is involved in cytosine hydroxylation, demethylation, and epigenetic regulation of gene expression [124]. In a fraction of acute myeloid leukemia (AML) patients, increased cytosines' methylations are caused by mutations in isocitrate dehydrogenase 2 (IDH2). Hence, L-ASC promotes differentiation and cell death specifically in AML with IDH2 mutations [125].

7. Augmented prooxidant therapy (APoT)

Augmented prooxidant therapy (APoT) is a ROS-based anticancer strategy, in which a prooxidant is combined with an inhibitor of antioxidant enzyme(s) [126]. Such combination works synergistically, causing a significant cellular accumulation of ROS, mainly H_2O_2, which cannot be effectively neutralized due to the induced impairment of the antioxidant system, resulting in rapid cancer cell death. The concept of APoT is depicted in Fig. 2. Noteworthy, growing evidence suggests that APoT seems to be highly selective for cancer cells, especially if the dependence of a given cancer on the particular antioxidant molecule is properly defined [70]. Therefore, APoT has a potential as a novel, effective and safe anticancer strategy. Examples of experimental APoT strategies employed so far in preclinical models are summarized in Table 1. Combination treatment modalities involving selected ROS-generating compounds, and in particular those involving L-ASC as a prooxidant, are presented below.

7.1. Downregulation of antioxidant enzymes combined with ROS-generating agents

The first experimental APoT approaches involved the genetic downregulation of antioxidant enzymes. He et al. [127] demonstrated that the knockdown of PRDX1 increased the sensitivity of A549 (human non-small lung cancer) or HeLa (human cervical cancer) cells to ROS-generating quinone, vitamin K3 (menadione, MEN). In contrast, this combination was significantly less or non-toxic for non-cancerous HUVEC cells or primary normal fibroblasts [127]. Interestingly, enhanced, oxidative-stress mediated cytotoxicity of PRDX1-deficient HeLa cells was observed upon exposure of these cells to β-lapachone, another quinone able to induce cellular ROS production [128]. Similarly, downregulation of PRDX1 potentiated the toxicity of other ROS-generating agents, glucose oxidase (GOx) or L-ASC in breast cancer cells *in vitro* [70] and both, *in vitro* and *in vivo* in malignant B cells [71]. Importantly, the non-malignant, immortalized mammary cells with downregulated or inactive PRDX1 were markedly more resistant to GOx or L-ASC exposure [70]. Also, genetic downregulations of other antioxidant enzymes such as SOD2 [120], prostaglandin reductase 1 (PTGR1) [91], and TXNRD1 [71] sensitized malignant cells to L-ASC, confirming their role in H_2O_2 scavenging.

7.2. Combination of TXNRD inhibitors and L-ascorbate

Recently, it was demonstrated that AUR, an inhibitor of TXNRD1/2, potentiates anti-cancer activity of pharmacological L-ASC *in vitro* and *in vivo* against B cell malignancies [71] as well as breast cancer cells [91]. AUR-mediated inhibition of TXN-dependent H_2O_2 removal greatly sensitized malignant B-cell-derived cell lines, as well as primary chronic lymphocytic leukemia (CLL) cells, but not normal B cells, to L-ASC *in vitro*. The *in vivo* efficacy of the combination tested to human lymphoma B cells xenografted to immune-deficient mice was moderate. Investigation of the mechanism of the synergistic cytotoxicity of the combination revealed that H_2O_2 extracellularly generated by L-ASC diffuses through the cell membranes and rapidly accumulates in malignant B cells, as a result of limited ROS-scavenging capacity of the cells due to AUR-mediated inhibition of the TXN system. Both the oxidative macromolecule damage and the cytotoxic effect were greatly dependent on the intracellular iron pool [71].

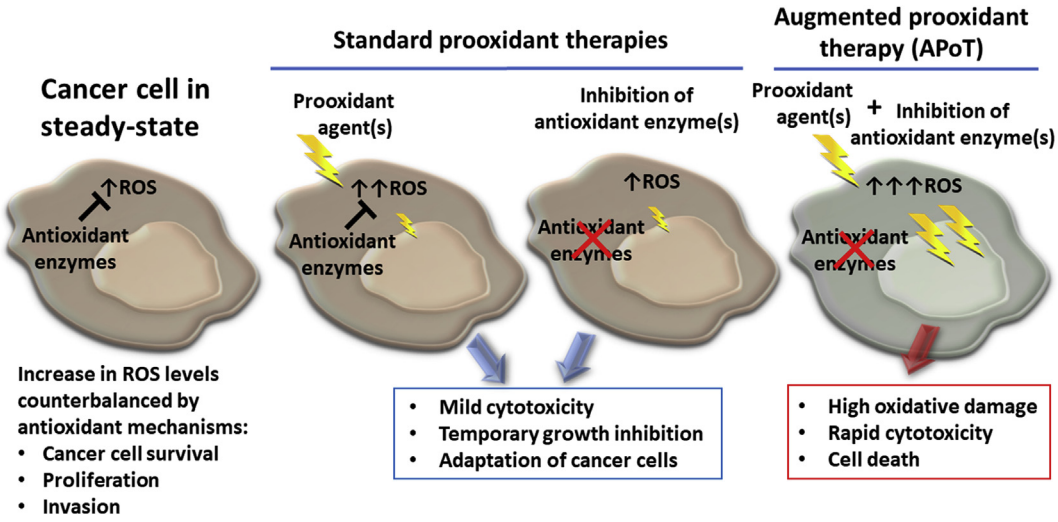

Fig. 2. Scheme representing the concept of augmented prooxidant therapy in cancer. A balance between reactive oxygen species (ROS) production and antioxidant defense promotes metabolic changes and cancer cell survival (left panel). Standard prooxidant therapies only temporarily inhibit tumor growth, eventually leading to cancer cell adaptation (middle panels). Conversely, the simultaneous application of prooxidant agent(s) with inhibition of ROS-neutralizing enzymes or depletion of the small molecule antioxidants can lead to immediate killing of cancer cells (right panel).

Table 1
Examples of anticancer APoT-type strategies tested in preclinical models.

Prooxidant compound	Type of the compound	Antioxidant inhibitor	Type of human cancer cells sensitive for the APoT strategy	Research phase/model	Reference
L-ASC	furanone	AUR (inhibitor of TXNRD1/2)	B cell malignancies, triple-negative breast cancer (TNBC) cells	*In vitro* and *in vivo* in murine model	[71] [91]
		PRDX1 knockout	TNBC, B cell malignancy	*In vitro* and/or *in vivo* in murine model	[70] [71]
		SOD2 knockout	Non-small-cell lung carcinoma (NSCLC) cells	*In vitro*	[139]
		PTGR1 knockdown	NSCLC, TNBC	*In vitro*	[91]
		TXNRD1 knockdown	B cell malignancy	*In vitro*	[71]
		Arsenic trioxide (ATO)	Hematological malignancies	*In vitro* and *in vivo* in murine models/clinical trials	[132] [133] [134] [135] [136]
MEN	quinone	PRDX1 knockdown	NSCLC, cervical adenocarcinoma cells	*In vitro*	[127]
β-lapachone	quinone	PRDX1 knockdown	cervical adenocarcinoma cells	*In vitro*	[128]
MEN and L-ASC		Pre-treatment with BSO	Breast cancer and prostate cancer cell lines	*In vitro*	[140]
Curcumin (highly bioavailable form)	polyphenol	ES936 (an inhibitor of NAD(P)H quinone dehydrogenase 1)	esophageal squamous cell carcinoma	*In vitro* and *in vivo* in murine model	[141]
WZ35, a curcumin analogue	polyphenol	Cisplatin (inhibitor of TXNRD1/2)	gastric cancer cells	*In vitro* and *in vivo* in murine model	[142]
Etoposide/rotenone/ curcumin	polyphenols	Flavonoids inducing GSH depletion (e.g. hydroxychalcone, chrysin, apigenin)	NSCLC, leukemia and prostate cancer cells	*In vitro*	[143]
Quercetin	polyphenol	ATO	Myeloid leukemia cells	*In vitro*	[144]

The potent antitumor efficacy of the AUR + L-ASC combination towards triple-negative breast cancer (MDA-MB-231) cells *in vitro* and *in vivo* has been also recently reported [91]. The combined treatment differentially affected various cancer cell lines, and the in-depth proteomic and genetic analyses revealed that the upregulation of selected cellular antioxidant systems confers resistance to the AUR + L-ASC combination. In particular, the high expression level of PTGR1 most significantly correlated with increased resistance to the combined treatment [91]. Importantly, the treatment exerted a minor toxic effect toward non-cancerous cells.

Several lines of evidence support that the extracellularly generated H_2O_2 is crucial for the cytotoxicity of the AUR + L-ASC combination to malignant cells. First, D-ASC, which does not enter cells as efficiently as L-ASC due to transporter stereo-selectivity, in combination with AUR triggered even more potent cytotoxic effect to malignant B cells [71]. Second, L-DHA, a reduced form of L-ASC that generates ROS intracellularly, was ineffective [71]. Finally, the cytotoxic effect of AUR + L-ASC could be entirely abolished by the addition of CAT [71] or pegylated CAT [91] to the culture medium.

7.3. Combination of arsenic trioxide and L-ascorbate

Long before the synergism between L-ASC and AUR was discovered, it had been shown that L-ASC potentiates the anticancer activity of arsenic trioxide (ATO). ATO is approved for the treatment of relapsed acute promyelocytic leukemia [129]. The mechanism of action of ATO is complex and involves numerous alterations in cellular homeostasis, including inhibition or downregulation of antioxidant enzymes and affecting redox signaling molecules [40,82,130,131]. The synergistic effects of ATO and L-ASC were shown in AML and B-cell lymphoma cell lines, as well as in primary CLL. Furthermore, ATO combined with L-ASC prolonged the survival of P388D lymphoma-bearing mice [132]. The following *in vitro* studies showed that L-ASC in association with ATO selectively kill primary human multiple myeloma cells [133]. Noteworthy, normal bone marrow cells or normal hematopoietic progenitor cells displayed modest sensitivity to the treatment [133,134]. However, the combination of ATO with L-ASC provided limited clinical benefit to patients with hematological malignancies [135,136]. Clinical trials testing the efficacy of ATO and L-ASC in combination with other drugs (e.g., melphalan or bortezomib) revealed more promising results [137,138]. In summary, although ATO + L-ASC may be considered a prototype of APoT, its limited clinical efficacy can be explained by the complex mechanism of ATO antitumor activity, as well as by significant, dose-limiting toxicity of the drug.

8. Conclusions

The maintenance of equilibrium, with only small fluctuations between production and removal of ROS is indispensable for the proper functioning of the cell and the organism as a whole. Many types of cancer cells have hijacked this potential for protecting their integrity under the conditions of intrinsically exaggerated overproduction of ROS. Importantly, however, these excessive oxidative stress conditions can also be regarded as an “Achilles heel” of cancer and utilized for therapeutic use. This treatment must be strong enough to lead to the death of cancer cells but in hope of sparing the healthy tissue and assuming that the latter is characterized by dramatically lower starting levels of ROS. It is usually achieved by either inducing a rapid increase of ROS delivery into tumor cells or an abrupt inhibition of antioxidant defenses in cancer. However, when both approaches are combined, which is hereby referred to as APoT, they were shown to produce a potent synergistic effect. While the augmented prooxidant approach is still in its early stages in terms of clinical applications, it may pose an attractive alternative or addition to classical chemotherapies of cancer in the future. Still, the therapeutic efficiency, dozing, and safety of such combinations need to be ascertained on a larger scale in pre-clinical studies using animal models, including immunocompetent models, before it is broadly tested in patients.

CRediT authorship contribution statement

Malgorzata Firczuk: Writing - original draft, Writing - review editing. **Malgorzata Bajor:** Writing - original draft, Writing - review editing. **Agnieszka Graczyk-Jarzynka:** Writing - original draft, Writing - review editing. **Klaudyna Fidyt:** Writing - original draft, Writing - review editing. **Agnieszka Goral:** Writing - original draft,

Writing - review editing. **Radoslaw Zagozdzon:** Writing - original draft, Writing - review editing.

Declaration of competing interest

Radoslaw Zagozdzon, Malgorzata Firczuk, Agnieszka Graczyk-Jarzynka, and Malgorzata Bajor are co-inventors on the patent granted by European Patent Office entitled “Synergistic combination of TXNR inhibitors and ascorbate for treatment of B cell malignancies” (Publication No. EP3181118B1).

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