Conference paper

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Anti-cancer agents and reactive oxygen species modulators that target cancer cell metabolism

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Abstract: Traditionally the perspective on reactive oxygen species (ROS) has centered on the role they play as carcinogenic or cancer-causing radicals. Over the years, characterization and functional studies have revealed the complexity of ROS as signaling molecules that regulate various physiological cellular responses or whose levels are altered in various diseases. Cancer cells often maintain high basal level of ROS and are vulnerable to any further increase in ROS levels beyond a certain protective threshold. Consequently, ROS-modulation has emerged as an anticancer strategy with synthesis of various ROS-inducing or responsive agents that target cancer cells. Of note, an increased carbohydrate uptake and/or induction of death receptors of cancer cells was exploited to develop glycoconjugates that potentially induce cellular stress, ROS and apoptosis. This mini review highlights the development of compounds that target cancer cells by taking advantage of redox or metabolic alteration in cancer cells.

Keywords: cancer cell metabolism; glycoconjugate; ICS-28; reactive oxygen species; ROS-modulator.

Introduction

Most cancer cells undergo metabolic reprogramming that support their survival and proliferation. This metabolic reprogramming plays a pivotal role in redox homeostasis in cancer cells [1–3]. These metabolic activities include aerobic glycolysis and pentose phosphate pathway (PPP) production of NADPH [4]. NADPH is also synthesized by malic enzyme 1, isocitrate dehydrogenase and one-carbon metabolism [5]. NADPH produced by the above pathways is an essential reducing equivalent for protection against reactive oxygen species (ROS) in cancer cells [5]. This is facilitated by glycolytic enzymes such as the M2 isoform of pyruvate kinase (PKM2) which diverts metabolites into PPP [6]. The PPP produce ribose-5-phosphate, required for nucleotide biosynthesis and critical for cell proliferation [5, 6]. Cancer cells also enhance glucose-uptake to fuel these enhanced metabolic activities [2]. High abundance of glucose in the cytoplasm of a cancer cell also increases flux into other metabolic pathways such as hexosamine biosynthetic pathway (HBP) [7].

Furthermore, the Krebs cycle intermediates are replenished by uptake of glutamine, which leads to increased production of amino acid precursors [8]. Redirection of glutamine to cellular energetics destabilizes glutathione homeostasis in glioblastoma cells and other cancer cells [9]. Indeed, increased glutamine catabolism is a hallmark of tumor metabolism reprogramming [9]. Other studies report that glutaminolysis inhibition is associated with depletion of GSH and ROS generation [10]. Furthermore, serine biosynthesis increases

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glycine availability, which is vital for glutathione biosynthesis [11]. This indicates the intricate control of metabolism and redox balance for survival of cancer cells that can be targeted by therapeutic agents.

Over the years, it has emerged that disruption of cancer cell metabolism and increasing ROS beyond cancer cell antioxidant protective threshold is an effective strategy of selective killing of cancer cells [12–15]. This is because cancer cells maintain high basal level of ROS, thus they are vulnerable to any further increase in ROS (Fig. 1). Moreover, multiple sources of ROS in a cancer cell make it possible for ROS species to effect damage to various macromolecules; including DNA, proteins and lipids [16, 17]. Furthermore, several cancer chemotherapeutic agents, such as cisplatin and paclitaxel induce apoptosis partially via ROS generation and by upregulation of death receptors [18–21]. A review on therapeutic targeting of cancer via death receptors dissects this topic comprehensively and is therefore subtly discussed in this minireview which focuses on ROS cancer therapeutics [22].

Altered cancer cell metabolism and cancer cell targeting

Metabolic reprogramming in cancer cells enhance carbohydrate uptake necessary to fuel anabolic activities that support cell proliferation [23, 24] and suppress apoptosis. High glucose uptake in cancer cells vs normal cells is facilitated by overexpression of glucose transporters such as GLUT-1, GLUT-2, GLUT-3 and GLUT 4 [2]. GLUT-1 overexpression is oncogene driven by mutated genes such as Ras and Raf [2]. Moreover, it has been shown using light diffraction studies in human red blood cells that several sugars, including 2-deoxy-D-glucose, D-mannose, D-galactose, D-xylose, 2-deoxy-D-galactose, L-arabinose, D-ribose, D-fucose, and D-lyxose are transported by GLUT-1 [25].

D-galactose, has been reported to possess an equivalent GLUT-1-mediated uptake rate as D-glucose [25]. D-galactose can enter glycolysis through phosphorylation and epimerization reactions that convert it into glucose-1-phosphate, and then to glucose-6-phosphate through the action of phosphoglucomutase [26]. Moreover, biological interconversion of D-galactose and D-glucose takes place by the Leloir pathway and requires three enzymes galactokinase, galactose-1-P uridylyltransferase, and UDP-galactose 4-epimerase [27]. On the other hand, D-mannose can enter the glycolytic pathway through phosphorylation to mannose-6-phosphate and isomerization to fructose-6-phosphate by phosphomannose isomerase [28]. However, D-arabinose, L-fucose, L-glucose, L-xylose and L-rhammose are poor GLUT-1 substrates [25].

This broad utilization and differential rate of monosaccharide uptake in cancer cells compared to normal cells has been used in positron emission tomography (PET). In PET, 2-[18F]-2-Deoxy-D-glucose (FDG) is used in imaging and locating tumor metastases [29]. Concomitantly, 2-deoxy-D-glucose is used as an anticancer agent. Broad specificity and high monosaccharide uptake by GLUT-1 is used in targeting

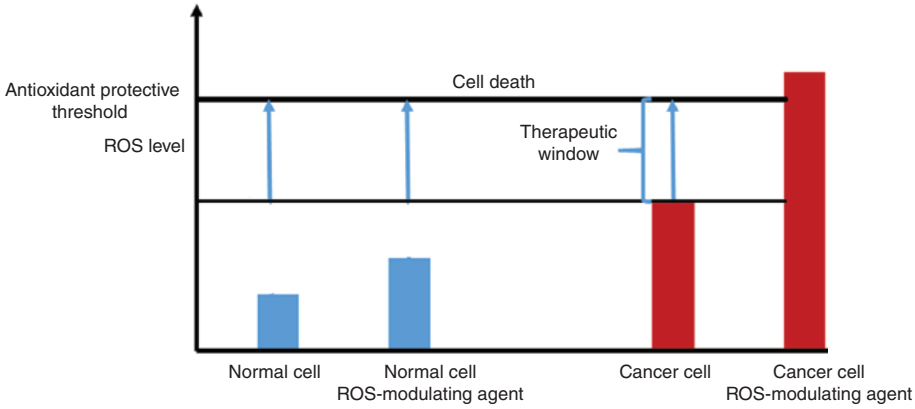

Fig. 1: ROS-modulation in normal vs. cancer cell.

cancer cell metabolism. Indeed, substitution at various positions on the glucose moiety is well tolerated by GLUT-1 [30–32]. Furthermore, several groups have investigated the roles of glucose and glucose-deprivation, 2-deoxy-D-glucose and glycoconjugated anticancer agents in ROS elevation, death receptor signaling and cancer cell metabolism [22, 33–37]. This review traces salient features of such studies.

Glucose and glucose deprivation

Cancer cells show higher susceptibility to glucose-dependent ROS generation and glucose deprivation-induced cytotoxicity compared to normal cells [3, 4, 6, 38–42]. This higher susceptibility to glucose deprivation is due to high demand for glucose necessary for generating reducing equivalents in PPP [39, 40, 43]. Moreover, augmentation of antioxidant machinery in many cancers such as colon and breast cancer have been shown to be a response to high rates of $O_2^{\cdot-}$ and H_2O_2 due to mitochondrial mutations [44]. One study showed that mutations of fumarate hydratase in a hereditary leiomyomatosis renal cancer cell line (HLRCC) led to an increase of glucose-mediated ROS generation [45].

Additionally, hypersensitivity of cancer cells to glucose-deprivation is promoted by oncogenic pathways such as Akt/mTOR pathway [46]. Glucose-deprivation inactivates mammalian target of rapamycin (mTOR) which downregulates protein synthesis and contribute to cancer cell killing [47]. These oncogenic pathways render cells unable to stop anabolism thus making glucose-deprivation induce oxidative stress responses and cell death [42, 46, 48, 49]. In addition, cancer cells are sensitive to death receptor-mediated apoptosis by glucose-deprivation or by 2-DG [22, 49].

In normal cells, low glucose manifests itself as low ATP/AMP ratio that is detected by adenosine monophosphate-activated protein kinase (AMPK) [50, 51]. AMPK stabilizes p53 to cause cell cycle arrest [51]. Conversely, most cancer cells have mutant p53 that facilitate cell proliferation. However, small molecules that mimic glucose-deprivation induce ROS elevation which activate AMPK that stabilize p53, triggering cell cycle arrest [15, 51–53]. In response to glucose-deprivation, AMPK also promotes fatty acid oxidation, which is a ROS-generating biochemical process [54]. Additionally, lack of glucose is detected by the carbohydrate response element-binding protein (ChREBP) which is a transcription factor that deactivates genes that regulate glycolysis [55]. Cells in which ChREBP is suppressed activate p53, show cell cycle arrest and in vivo reduction in tumor growth [55].

Furthermore, glucose-deprivation generate ROS which cause endoplasmic reticulum (ER) stress that induce unfolded protein response (UPR) [56]. Unfolded proteins accumulate because glucose-deprivation conditions do not provide sufficient glucose necessary for glycosylation-dependent protein folding [57]. Moreover, UPR is impaired in cancer and is controlled by a glucose-stimulated protein called glucose-regulated protein-78 (GRP-78) which is considered a biomarker of ER stress [54, 58–61]. These data suggest that glucose-deprivation disruption of cancer metabolism and ROS generation may be used to control cancer cell proliferation.

2-Deoxy-D-glucose (2-DG)

2-DG is a glucose analog in which the C-2 hydroxyl group is replaced with hydrogen. 2-DG competes with glucose for uptake via glucose transporters such as GLUT-1 [62, 63]. 2-DG is also a competitive inhibitor of hexokinase (HK) [62–65]. Phosphorylation of 2-DG by HK sequesters it within the cytoplasm and is not metabolized further in the glycolytic pathway [63]. This reduces availability of PPP metabolites such as NADPH that is involved in generation of reducing equivalents such as glutathione. The net effect is increase in ROS. In this way, 2-DG is a ROS modulator that is cytotoxic in many cancer cell types [64, 65]. 2-DG also mimics glucose-deprivation through AMPK activation, mTOR inactivation and cell cycle arrest [62].

Many studies have demonstrated 2-DG cytotoxicity and increase in ROS that is ablated by thiol antioxidants such as N-acetylcysteine [63]. In one study, a 50 % reduction of the glycolytic rate was observed in

LNCaP prostate cancer cells upon a 2-h treatment with 5 mM 2-DG [66]. Accumulation of phosphorylated 2-DG kills the cancer cell because several reports indicate that 2-DG augments generation of ROS [67]. However, a new metabolic fate of 2-DG in cancer involving glucose-6-phosphate dehydrogenase within endoplasmic reticulum and utilizing NAD and NADP has been proposed and investigated [68].

There is also evidence that GCL activity is upregulated upon 2-DG cell treatment suggesting that GCL is involved in protecting cancer cells from 2-DG induced ROS [67]. Moreover, competition between glucose and 2-DG mimics glucose-deprivation conditions with ROS-generating effects described above. In another study, combination of 2-DG and 6-amino nicotinamide, induced ROS and sensitized cancer cells to radiotherapy through multiple cell death pathways [69]. Indeed, one clinical trial showed that the combination of 2-DG with radiation was well tolerated in cancer patients [70]. 2-DG is also more effective in combination therapies as has been demonstrated in 2-DG/paclitaxel for non-small lung cancer, 2-DG/doxorubicin for human osteosarcoma and 2-DG/Mito-CP for breast cancer [71, 72].

The mechanisms being studied to explain 2-DG cytotoxicity include increased oxidative and ER stress, disruption of cellular energetics, interference with N-linked glycosylation, and induction of death receptor -mediated apoptosis, autophagy, necrosis and mitotic catastrophe [73]. Apoptosis is a form of cell death promoted by caspases which degrade cellular substrates without extracellular cytoplasmic spillage [74]. Indeed, 2-DG sensitized human melanoma cells to TRAIL-induced apoptosis by up-regulation of TRAIL-R2 via the unfolded protein response [75]. Conversely, necrosis involves rupture of the plasma membrane and cell death. These cell death mechanisms have fueled the use of 2-DG in combination with anticancer agents and with radiotherapy.

Bleomycin

Bleomycin is a glycoconjugate (Fig. 2) used to treat various forms of cancer such as Hodgkin's lymphoma, testicular, ovarian and cervical cancers which acts by induction of DNA strand breaks [76]. The anti-cancer agent bleomycin was first purified from *Streptomyces verticillus* cultures and approved by the FDA in 1973. The exact mechanism of DNA strand break is unclear but it has been suggested that bleomycin complexes with copper(II) and iron(II) ions to produce an intermediate [77] that reacts with oxygen to produce O_2^- and $HO^{\cdot}$ free radicals that cleave DNA. In addition, these complexes also mediate lipid peroxidation and other macromolecules [77]. The sugar moiety appears to be important for ROS elevation and cytotoxicity. For example, when tested on HEp-2 laryngeal carcinoma cells, deglycosylated bleomycin decreased cell viability and clonogenic survival, but was less toxic than carbohydrate containing bleomycin. In addition, deglycosylated bleomycin induced apoptosis, independent of ROS production and caspase activation in laryngeal carcinoma cells [78]. However, bleomycin-induced pulmonary damage and fibrosis in patients undergoing treatment with this drug is a major drawback [76].

Etoposide

Etoposide is an FDA approved cancer drug and is a glycoconjugate of podophyllotoxin (Fig. 2) with a D-glucose derivative [79–81]. Podophyllotoxin is derived from the rhizome of the wild mandrake (*Podophyllum peltatum*). Etoposide generates ROS, interferes with the breakage-reunion reaction mechanism of topoisomerase II and is used in treatment of testicular cancer, Ewing's and Kaposi's sarcoma, lung cancer, leukemia, and glioblastoma multiforme [80, 81]. In one study, an increase in ROS was observed in Daudi and Raji cells treated with etoposide [80]. This increase in ROS was ablated by the antioxidant NAC with concomitant reduction in apoptosis. Additionally, increased expression of death receptors 4 (DR4) and 5 (DR5) synergizes the apoptosis response to combined treatment with etoposide and TRAIL [82].

Moreover, protein kinase C (PKCδ) overexpression enhances the sensitivity of neuroblastoma cells to etoposide-induced ROS production and apoptosis [83]. In the study, PKCδ transfection and overexpression

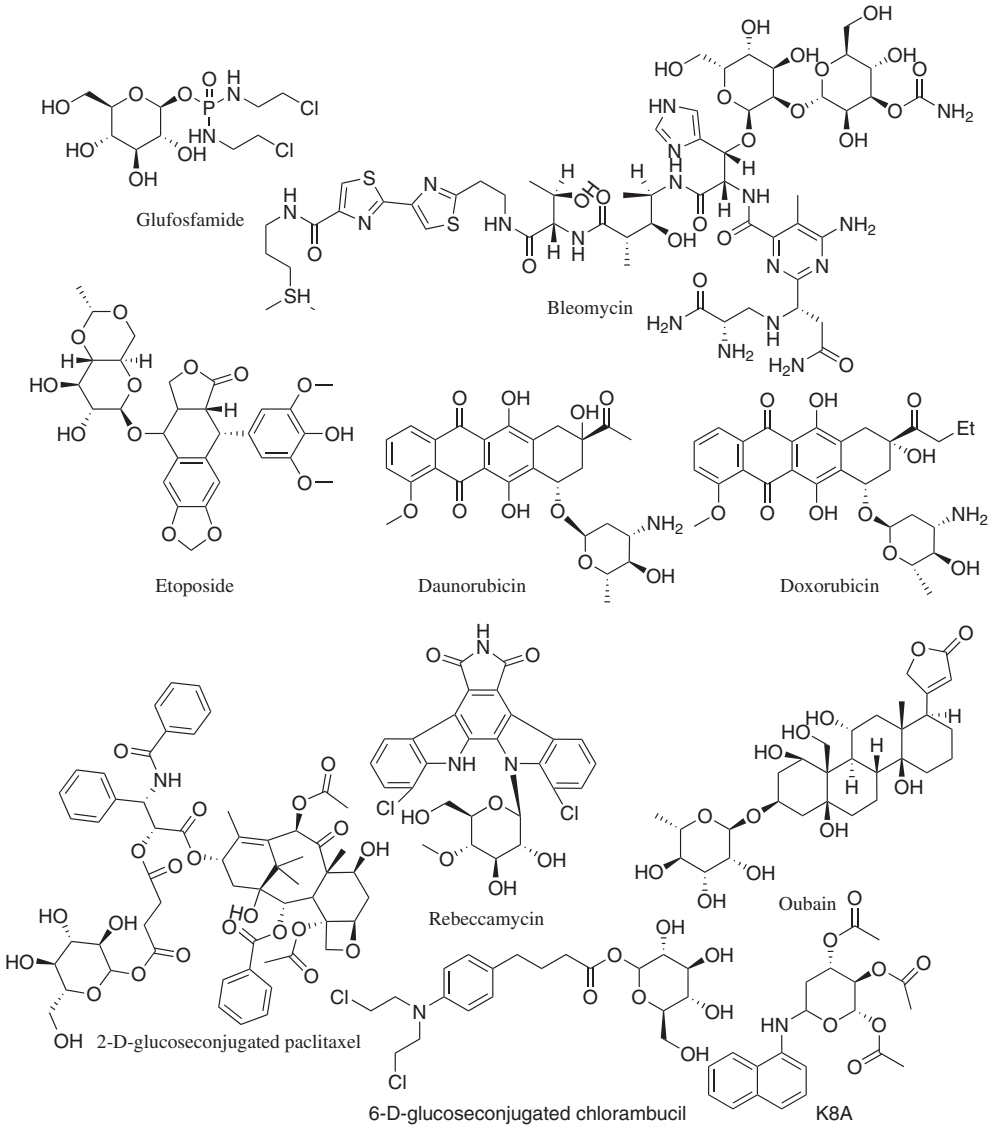

Fig. 2: ROS-modulating glycoconjugates.

induced ROS overproduction leading to apoptosis of BSO-resistant NB cells [83]. However, a recent phase II clinical trial combination study with cisplatin did not improve beneficial outcomes and showed increased deleterious cytotoxicity in patients [81].

Doxorubicin

Doxorubicin is an anthracycline that is used alone or in combination with other medications to treat several different types of cancer. For example, doxorubicin in combination therapy sensitizes renal cell carcinoma cells to death receptor 4 (DR4)-mediated apoptosis with activation of caspases [84, 85]. Doxorubicin is a glycoconjugate (Fig. 1) whose planar aromatic part of the molecule intercalates between two DNA base pairs whereas the six-membered daunosamine sugar positions itself in the minor groove [86]. In this way, doxorubicin inhibits the progression of topoisomerase II which relaxes supercoils in DNA for transcription. Furthermore, this interaction may lead to DNA breaks which impairs replication. These DNA strand breaks induce

ROS-generating pathways which contribute to doxorubicin cytotoxicity. The daunosamine sugar moiety is important for doxorubicin activity because 7-deoxydoxorubicionolone lacking the sugar has been shown to be many times less toxic than doxorubicin [87–89].

Reduction of doxorubicin by mitochondrial reductases generate unstable anthracycline semiquinone free radicals which reduce molecular O_2 to O_2^- [90]. In addition, several enzymes, including cytochrome p450, NAD(P)H dehydrogenase, and endothelial nitric oxide synthase, have been implicated in the reductive metabolism of doxorubicin [90, 91]. However, a recent study, demonstrates the impact of NF-κB pathway gene expression on the prognosis of triple-negative breast cancer (TNBC) and suggest that SP1 gene expression level is a prognostic marker in TNBC patients receiving adjuvant doxorubicin chemotherapy [92]. Sp1 expression promotes cell growth, cell survival and gene expression. In addition, Sp1 overexpression induces doxorubicin resistance in a myeloid leukemia cell line called HL-60 [92].

Furthermore, doxorubicin has been implicated in cardiotoxicity that manifest during treatment and long after initial administration [90–93]. In addition, recent studies indicate that doxorubicin inhibits XBP-1 activation thus interfering with UPR [59]. Similar effects have been observed with daunorubicin, a doxorubicin analog [90, 91].

Glucose-conjugated ifosfamide

Glufosfamide is a glucose conjugate of ifosfamide in which isophosphoramide mustard, the alkylating metabolite of ifosfamide, is linked to the β-D-glucose molecule. The glucose moiety of glufosfamide enhances uptake by GLUT-1 before being cleaved by glucosidases to ifosfamide to effect cytotoxicity [94, 95]. Ifosfamide is a DNA-alkylating agent with anticancer properties [94] and a structural analog of cyclophosphamide. Glufosfamide is undergoing phase III clinical trials at NCI. Phase III is designed to assess whether glufosfamide provides additional survival benefit as compared to 5-fluoro-uracil in patients with metastatic pancreatic cancer [94].

Glufosfamide inhibits the synthesis of DNA and protein in a breast carcinoma cell line MCF7, as demonstrated by low incorporation of [3H-methyl]-thymidine into DNA and [14C]-methionine into protein of these cells [95, 96]. In an earlier study, glufosfamide inhibited the growth of MiaPaCa-2 tumors in an orthotopic nude mouse model [97]. This inhibition in growth is enhanced in combination with gemcitabine and other anticancer agents [98].

Glucose-conjugated paclitaxel

Paclitaxel (taxol) is a diterpenoid taxane derivative and a ROS generating anticancer drug used in treatment of breast carcinomas and other cancers. Paclitaxel binds tubulin, disrupts cell division and induces cancer cell death. In cell culture, exposure of A549 lung cancer cells to paclitaxel within 5 μM for 15 min increased ROS [99]. Paclitaxel also kill cancer cells via death receptor independent pathways via caspases-3 and -8 activation [100].

To improve selectivity and water solubility of paclitaxel, glucose-conjugation has been employed in the synthesis of four paclitaxel based prodrugs [101]. These prodrugs showed reduced toxicity toward normal cell lines but were more effective in GLUT-overexpressing cancer cells. These data suggest that carbohydrate-based paclitaxel prodrugs are potential candidates for cancer therapy because they utilize the high sugar uptake and high ROS phenotype observed in most cancer cells.

Glucose-conjugated chlorambucil

Chlorambucil is a nitrogen mustard alkylating agent which is a close structural congener of melphalan. It attaches the alkyl group to the guanine base of DNA on the seventh nitrogen atom of the imidazole

ring [102, 103]. In addition, treatment of primary chronic lymphocytic leukemia cells with chlorambucil increased cell surface expression levels of death receptor-4 (DR4) and DR5 and apoptosis [102–104]. Glucose-conjugation improves water-solubility and previous work suggests that sugar conjugation does not interfere with chlorambucil-mediated alkylation of DNA [102, 103]. Glucose-conjugated chlorambucil via a C6 ester or amide bond promotes glucose-deprivation by competitively inhibiting the uptake of glucose by GLUT-1 in human red blood cells [102]. Furthermore, one ester-linked compound, 6-D-glucose-conjugated chlorambucil (Fig. 1) had more than 160-fold inhibition activity on the entry of radiolabeled glucose than GLUT-1 substrate, D-glucose [102]. The inhibition activity was reversible suggesting that it is not due to nucleophilic alkylation of a GLUT-1 moiety. Such glucose-deprivation conditions enhance ROS generation.

In another study, chlorambucil was conjugated to FDG to generate 19 novel potent glycoconjugates with structurally diverse linkages at the anomeric carbon [103]. The cytotoxic activities of these glycoconjugates were screened in six human tumor cell lines; ovary adenocarcinoma (PA1) breast adenocarcinoma (MCF-7), colon adenocarcinoma (DLD-1), prostatic adenocarcinoma (PC3), lung cancer (A549), melanoma (M4Beu) and a primary culture of human fibroblasts [103].

Oubain

Oubain is a cardiac glycoside that inhibits the Na^+/K^+ ATPase by binding to its alpha-subunit. Inhibition of Na^+/K^+ ATPase impairs the activity of the Na^+/Ca^{2+} exchanger (NCX) leading to accumulation of Ca^{2+} followed by cancer cell death [105]. The activity and potency of oubain rely on the rhammose sugar moiety since the aglycone oubagenin binds poorly on the Na^+/K^+ ATPase [106]. Binding and inhibition of oubain begins with the binding of the hydrophobic core, which unlocks a binding site for the rhammose moiety [107]. The subsequent binding of rhamnose then holds the oubain molecule tightly to the sodium pump to halt pumping and induce accumulation of Ca^{2+} and generation of ROS [106, 107].

Oubain has been shown to increase ROS generation in various cell lines. One study reports that Oubain causes two-fold increase in ROS in U937 cells compared to untreated cells at 1 μM concentration [106]. However, in HT29 cells, oubain increased cytosolic Ca^{2+} concentration, increased ROS and enhanced the activity of the calmodulin kinase II enzyme, which in turn activate hypoxia-inducible factor-1 alpha (HIF1α) [107] which regulate many cellular activities, including glucose metabolism.

Rebeccamycin

Rebeccamycin is an indolocarbazole (ICZs) which are a class of compounds with an indole and carbazole backbone that are under current study due to their potential as anti-cancer drugs. Rebeccamycin is a weak topoisomerase I inhibitor with antitumor properties against mouse B16 melanoma cells and against P388 leukemia cells [108] and other cancer cell types. Rebeccamycin also inhibits SR protein kinase activity of topoisomerase I [108, 109]. Type I topoisomerases are enzymes that cut, relax and reanneal one of the two strands of double-stranded DNA.

The carbohydrate moiety position is extremely important for topoisomerase I inhibition. The deglycosylated form, deschloro-rebeccamycin aglycone has very low antimicrobial activity suggesting potency role of carbohydrate moiety [109]. The examination of effects on the cell cycle of L1210 cells suggest that in the absence of the sugar unit, the cells accumulate in the G1 phase but in the presence of sugar moiety the cells accumulate in the G2/M phases indicating different cellular targets. Compounds with a β-N-glycosidic bond, which fully intercalates into DNA, were more efficient at inhibiting topoisomerase I than their analogs with an α-N-glycosidic bond. Interestingly, Compounds with an α-*N*-glycosidic bond were stronger PKC inhibitors than compounds with a β-*N*-glycosidic bond [109]. However, various sugar moieties including, a glucopyranose, a galactopyranose or a fucopyranose attached to indolocarbazoles possessing a methyl group at the imide nitrogen showed no significant change in cytotoxicity [110].

Indolocarbazoles, and the anticancer glycoconjugates are however bulky with water solubility problems because of a large hydrophobic moiety (Fig. 2). In addition, their large size makes them poor substrates of GLUT-1 [37, 111]. Synthesis of these glycoconjugates is also multistep in nature, tedious and often expensive. Moreover, these anticancer glycoconjugates were designed to act as prodrugs with sugar cleavage required to achieve higher potency than their aglycones [37, 111]. Currently, there is an emerging redesign of antineoplastic small molecules that are less bulky, more stable, that generate ROS with substrate specificity for GLUT-1, with higher potency and better selectivity in cancer cells [112]. In this regard, we recently synthesized carbohydrate-based small molecules that have potential to induce oxidative and endoplasmic reticulum stress in cancer cells. One of the stress inducer was an N-aryl glycoside, K8A (Fig. 1).

K8A

We initially used an anti-glycolytic agent 2-DG, to target metabolism in a lung cancer cell line (H1299), to increase ROS and induce cellular stress. In addition, we considered that various carbohydrates can be used for interruption of cellular energetics and glycosylation patterns leading to oxidative and ER stress that cause cell death. In this regard, we designed, synthesized and evaluated a library of 46 *N*-aryl glycosides with diverse sugars for induction of ROS and cytotoxicity in H1299 [15] and A549 cancer cell lines. We identified K8A that induce about two-fold induction of ROS and is cytotoxic with an IC_{50} of less than 30 μM in H1299 cells. We further showed that K8A activates AMPK, and stabilizes p53 and induce a higher cytotoxicity than 2-DG in H1299 cell line. We are currently synthesizing and evaluating similar compounds as well as examining elevation of ROS as a strategy in cancer cell killing.

Elevation of ROS as a strategy for cancer cell targeting

Previous studies have shown that ROS are often increased in cancer cells relative to normal cells, and they contribute to initiation, progression and metastases of cancer. Multiple factors cause the elevation of ROS in cancer cells, including metabolic reprogramming, oncogene activation, cellular hypoxic conditions and Warburg effect [113].

For example, oncogene activation of MYC and KRAS facilitates generation of glycolytic metabolites, which flux into various biochemical pathways that promote cell proliferation while generating high ROS [113–115]. Also, RAS transformation activates *RAC* that regulates membrane-associated NADPH oxidase for generating O_2^- [115]. In addition, mitochondrial DNA mutations in cancer enhance ROS production and tumorigenesis [116, 117]. Thirteen polypeptides of the respiratory complexes are coded by mitochondrial DNA of which mutation may significantly affect the respiratory activity in mitochondria [118]. Moreover, mitochondrial DNA mutation may be enhanced due to its proximity to the site of ROS production within mitochondria, thus increasing its vulnerability to ROS-induced damage.

Overall, multiple mechanisms contribute to the elevation of ROS in cancer cells. Consequently, cancer cells enhance their anti-oxidant mechanisms to maintain ROS within a certain threshold level that affords their survival without detrimental damages [113]. This suggests that the redox system in cancer cells is finely tuned, and any insult or stress that further increase ROS beyond such antioxidant protective threshold (Fig. 1) cause cancer cell death [3]. Numerous studies have characterized the increased vulnerability or sensitivity of cancer cells toward ROS and the consequence of ROS-induced cancer cell death [119]. For example, ROS-induced death has been attributed to loss of superoxide dismutase (SOD3), catalase (CAT) and glutathione peroxidase 3 (GPX3) [119]. SOD3, CAT and GPX3 gene expression was examined in 1981 tumors covering 19 cancer types, showing that these antioxidant and other enzymes are differentially downregulated between cancer and normal cells [119].

Furthermore, recent studies have demonstrated that antioxidants, such as vitamin E and N-acetylcysteine, increase tumor progression, such as in mouse models of B-RAF and K-RAS induced forms of lung cancer [120]. Conversely, ROS-mediated oxidation and inactivation of the cell cycle phosphatase cdc25 contributes to cell cycle progression from G2 to M phase [121, 122]. It is therefore emerging that an elevation of ROS by using ROS-inducing compounds can be a strategy to tackle cancer cells with vulnerable redox systems [15].

ROS or redox modulators that target cancer cells

One of early ROS-generating alkylating antineoplastic agent is procarbazine (PCZ) or *N*-isopropyl-a-(2-methylhydrazine)-*p*-toluamide hydrochloride, was approved for human use in 1969 [123]. PCZ is used in treatment of various brain cancers and hodgkin's lymphoma. PCZ is an orally administered prodrug that requires extensive metabolism by cytochrome p450 enzymes for its activation. It is first oxidized to azoprocarbazine [124] and further oxidized to a mixture of methylazoxyprocarbazine and benzylazoxyprocarbazine isomers [125].

Over the years, many small molecules that can disturb the redox system were traditionally evaluated for their toxic effect toward cancer cells. Many of them are electrophilic or inhibit the redox systems, such as glutathione homeostasis. Consequently, many small molecules have been evaluated for ROS generation (Fig. 3) and antineoplastic activity in several cancer cell lines, in vivo and in clinical trials [126, 127]. Some of these small molecules are approved for patient use.

However, small molecule selectivity on GSH homeostasis enzymes is a subject of both interest and controversy in many studies because disruption of redox homeostasis may occur without necessarily activating ROS generation. Moreover, small molecule targets are diverse, effects are dissimilar and detailed mechanisms are unclear. Nevertheless, the role of ROS in cancer therapy is under rigorous study. We briefly review ongoing salient work of these ROS-generating non-carbohydrate small molecules.

Buthionine sulfoximine (BSO)

BSO is a synthetic peptide with a sulfoximine group (Fig. 3). BSO inhibits gamma-glutamylcysteine synthetase (also called gamma-glutamyl cysteine ligase, GCL) that catalyzes the rate-limiting step in glutathione

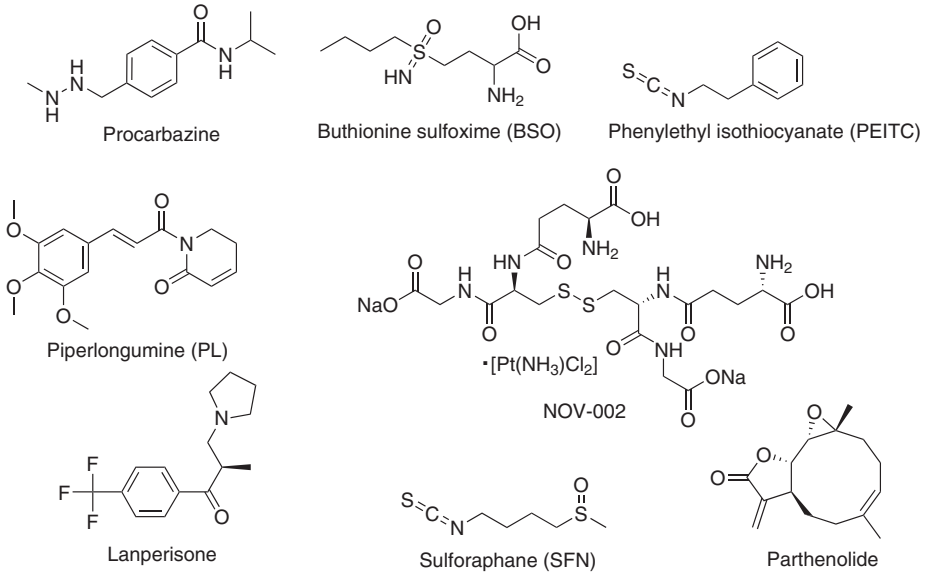

Fig. 3: ROS-modulators that target cancer cells.

biosynthesis [128]. BSO depletes an intracellular glutathione concentration and is used for increasing the sensitivity to many alkylating or cytotoxic agents. For example, an incubation of etoposide-resistant human MCF7 breast cancer cells (MCF7/VP) with BSO increases their sensitization to etoposide and vincristine [129].

The treatment of KU7, a bladder cancer cell line, with BSO leads to GSH depletion and enhances cisplatin ROS-generation and cytotoxicity. Also, cisplatin with inhibition of GSH biosynthesis by BSO selectively induced tumor regression in PI3K pathway mutant breast cancer cells both in vitro and in vivo [130]. BSO is in early clinical development at the National Cancer Institute (NCI) for the treatment of neuroblastoma in pediatric patients in combination with a DNA alkylating agent called melphalan [131]. Combination treatment with arsenic trioxide and BSO, increased the levels of DR5 and caspase-8 cleavage in NB4, U937, Namalwa, and Jurkat cells [132]. Together these data suggest the role of BSO in both ROS and death-receptor mediated apoptosis.

Phenylethyl isothiocyanate (PEITC)

PEITC is a natural substance found in cruciferous vegetables with an electrophilic isothiocyanate functional group (Fig. 3). PEITC reacts with thiol groups by non-enzymatic reaction with cysteine residues of proteins and by enzymatic conjugation with GSH, catalyzed by GSH S-transferases [133]. PEITC also inhibit glutathione peroxidase (GPX) and deplete GSH levels. These enzymatic and non-enzymatic reactions create a redox imbalance that induce apoptosis. Oncogenic transformation of HRAS increase ROS production, and treatment of HRAS-transformed ovarian epithelial cells with PEITC induced selective cytotoxicity [134]. In addition, animal studies show that PEITC has therapeutic activity in mice bearing Ras-transformed cells [135]. While PEITC can induce multiple effects due to its electrophilic property, its potency was decreased by addition of catalase and N-acetyl cysteine, which suggest that ROS production contributes to its mechanistic action for PEITC-mediated-cancer cell killing [135]. In addition, anti-proliferative effects of PEITC result from up regulation of DR4 and DR5 of TRAIL-mediated apoptotic pathways [136].

Sulforaphane (SFN)

Sulforaphane, like PEITC is a natural isothiocyanate (Fig. 3) extracted from cruciferous vegetables that inhibits cell proliferation in culture [137]. Sulforaphane induces ROS generation, is cytotoxic to hepatocellular carcinoma Hep3B cells [138] and downregulates expression of telomerase reverse transcriptase (hTERT). hTERT expression was restored upon treatment with the antioxidant N-acetylcysteine, thus implicating a ROS-mediated cell killing mechanism. In human osteosarcoma cells sulforaphane also upregulate TRAIL-induced apoptosis through the induction of DR5 [137, 139].

In another study, sulforaphane induced apoptosis and reduced viability in gemcitabine-sensitive pancreatic BxPC-3 cells but not in non-malignant ductal pancreatic cells [140]. Moreover, the evaluation of self-renewal by colony formation showed that cancer stem cell features were decreased in sulforaphane-treated cells than in gemcitabine resistant cells.

Interestingly, sulfoxythiocarabamate electrophilic tuning of sulforaphane yielded analogs that enabled a more elaborate understanding of the chemoprotective phase 2 enzyme induction mechanism of sulforaphane [141]. These analogs had inhibitory effect on lipopolysaccharide-induced nitric oxide formation like sulforaphane [141]. Together, these data suggest the complexity of the mechanisms of sulforaphane in ROS modulation in different cell lines and warrant further studies.

Parthenolide

Parthenolide, is a natural sesquiterpene lactone (Fig. 3) of the germacranolide class found in the feverfew plant (*Tanacetum parthenium*) with a pharmacologically important α-methylene-γ-lactone moiety [142].

Parthenolide generate ROS by activating NADPH oxidase [142]. Parthenolide also inhibit transcription factor, NF-kB by alkylating its p65-subunit at Cys 38 [143]. In one study, parthenolide induced apoptosis in pre-B acute lymphoblastic leukemia (ALL) cell lines by ROS generation that is characterized by loss of nuclear DNA, externalization of cell membrane phosphatidylserine, and depolarization of mitochondrial membranes at micromolar concentrations [144]. These data suggest parthenolide may have potential as a potent and novel therapeutic agent against pre-B ALLs and other cell lines [142, 144].

Furthermore, parthenolide sensitizes hepatocellular carcinoma cells to TRAIL by inducing the expression of death receptors [142, 145].

Recently, parthenolide analogs with IC_{50} values in the low micromolar range have been synthesized and evaluated in leukemia cells [146]. These C4 and C9 functionalized parthenolide analogs showed improved potency and selectivity towards acute myeloid leukemia (AML) cells versus normal human blood cells [146].

Lanperisone

Lanperisone, a muscle-relaxant piperidine derivative (Fig. 3) with cancer cell potency [147] was discovered by screening more than 50 000 compounds [148] using an ATP-based cell viability assay. Lanperisone acts by inducing iron-dependent oxidative stress called ferroptosis. Ferroptosis is a newly recognized form of regulated cell death characterized by mitochondrial morphological changes such as a small size, disappearance of crista and rupture of the mitochondrial membranes [149]. Ferroptosis is morphologically, biochemically and genetically different from other cell death pathways such as apoptosis, necrosis and autophagy.

LP increases ROS levels and selectively targets oxidatively stressed K-ras mutant cells via an LP-mediated iron and Ras/Raf/MEK/ERK signaling mechanism involving ferroptosis [148, 149]. In this study, LP induced non-apoptotic cell death in a cell cycle- and translation-independent way in oncogenic K-ras-expressing cells [147, 148]. Moreover, all ROS scavengers tested, including deferoxamine (an iron chelator), butylated hydroxyanisole (BHA), and the antioxidant trolox, a vitamin E analog, completely abolished cell killing by LP, strongly vouching for a ROS-mediated cell killing mechanism.

Piperlongumine (PL)

Piperlongumine is a natural electrophilic alkaloid bearing two α, β-unsaturated imides (Fig. 3) that selectively kills cancer cells by generation of ROS. PL is isolated from the long pepper plant (*piper longum*) [150]. PL was discovered using a cell-based small-molecule screening and quantitative proteomics approach [151]. The ROS-generating ability of PL is attributed to its multiple electrophilic sites that facilitate reaction with protein thiols and depletes glutathione in cells [152].

PL regulates GST Pi activity. Protein glutathionylation observed following PL treatment is not reversed by dithiothreitol treatment, pointing to existence of non-disulfide covalent attachments [153]. PL also promotes autophagy via inhibition of Akt/mTOR signaling to mediate cancer cell death [153]. Furthermore, PL and TRAIL synergistically act to upregulate DR5, which mediated TRAIL-induced apoptosis in many cancer cell types. Interestingly, this upregulation was found to be dependent on ROS and the activation of JNK and p38 kinases [150, 152, 154].

Interestingly, PL and HDAC inhibitor (vorinostat) hybrids are synergistic in potency and act by interfering with GSH levels and suppress DNA repair to induce apoptosis in leukemia cells (AML) [155]. More recently, the synthesis and structure/activity relationships of PL analogs has revealed sites responsible for increase of ROS and selective killing of cancer cells [153, 155, 156]. Indeed, one group successfully introduced an α-substituent chlorine on the lactam ring to moderately increase PL electrophilicity in the new analog PL-CL [157]. In comparison, PL-CL was a better ROS inducer and cytotoxic agent than PL, and showed more than 15-fold selectivity toward A549 cells over normal WI-38 cells [157].

NOV-002

NOV-002 is a non-toxic oxidized glutathione mimetic (Fig. 3) formulated with cisplatin at an approximately 1000:1 ratio, that alters the ratio of oxidized and reduced glutathione [158]. NOV-002-treated HL60 cells show increase in ROS and protein S-glutathionylation compared with untreated cells [159]. In a phase II clinical trial, one group investigated the addition of NOV-002 to standard preoperative chemotherapy in HER-2 negative breast cancer and in postmenopausal hormone receptor positive patients [160]. In this clinical trial, women with newly diagnosed stages II-IIIc HER-2 negative breast cancer received doxorubicin and cyclophosphamide followed by docetaxel and NOV-002. Interestingly, this treatment regimen was well tolerated and resulted in a very favorable pathologic completion response rate.

NOV-002 in combination with gemcitabine also reduces cancer cell invasion in vitro and metastasis in an animal model by regulating cell signaling pathways through suppression of ErbB2 and PI3K phosphorylation that induces inhibition of Akt and RhoA activation [161]. These data suggest that NOV-002 and other ROS modulators can be used in combination with anticancer agents.

Conclusion and future directions

Compelling evidence suggests that cancer cells have an altered mechanism and are under high ROS generation due to a complexity of factors that continue to be unraveled. Oncogenic signaling and metabolic reprogramming initiate and fuel apoptotic ROS-generating mechanisms in cancer cells. ROS-generation by cancer cells is an effective strategy of regulating cancer cell proliferation because high ROS is lethal above cancer-cell antioxidant protective threshold.

Moreover, ROS-generating small molecules may be designed to exploit high sugar avidity exhibited by most cancer cells. Together, high ROS-generation, high sugar uptake and altered metabolism represent effective targets that provide strategies for design and synthesis of carbohydrate-based antineoplastic agents that will continue to be explored by both academia and industry. Non-carbohydrate ROS-modulators that target death receptors offer a lot of promise particularly in combination with anticancer agents [20–22, 49, 75, 78, 82, 84, 85, 100, 104, 132, 136, 139, 145, 154]. The future in anticancer therapy probably lies in ROS-generating molecules that are potent and selective disruptors of cancer cell metabolism. In this regard, carbohydrate-based ROS-modulators together with electrophilic agents that target the antioxidant and death receptor mechanisms of cancer cells are major players.

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