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GLUTATHIONE: INDUCTION OF APOPTOSIS AND AUTOPHAGY IN CANCER

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ABSTRACT: Glutathione (GSH) is the most abundant non-protein thiol in eukaryotic cells capable of carrying out antioxidant defense mechanisms in the cell for its survivability, mostly against free radicals, *i.e.,* Reactive Oxygen Species (ROS). GSH protects normal cells against carcinogenic transformation, but high GSH levels in cancer cells decrease sensitivity to chemo- and radiotherapy, producing resistant cases of cancer. Hence, GSH depletion could be a potential mechanism by which resistant tumor cells can be sensitized to undergo apoptosis and autophagy. Thiol oxidation leads to the formation of permeability transition pore, promoting the extrusion of death-related molecular signals and activation of the intrinsic apoptotic pathway. Similarly, macroautophagic cell death involves the formation of auto-phagosomes that degrade organelles, including mitochondria, compromising normal cellular function and cellular death occurs by mechanisms such as mTorC1 inhibition. Therefore, the selective depletion of GSH in cancer cells could offer a promising approach in drug and radiation-resistant cases targeting multiple death-related cellular pathways. However, the selective depletion of such ubiquitous antioxidant, GSH in cancer cells yet remains to be a key challenge to the researchers worldwide.

INTRODUCTION:

Glutathione and Cell Homeostasis: Cells respond to various metabolic and internal stressors through highly regulated pathways. One such stressor is free radicals such as reactive oxygen species (ROS) including superoxide anions (O_2•−), hydroxyl radicals (OH•−), and peroxide radicals (ROO•) sources being mitochondria [1, 2]. In mitochondria, the electron transport chain (ETC) produces ROS as an inevitable by-product [3]. Complex I (NADH: ubiquinone oxidoreductase) and the mitochondrial cytochrome bc1 complex (complex III; ubiquinol: cytochrome c oxidoreductase) are the main producers of superoxide which are released into the intermembrane space (approx. 80% of the generated superoxide) or the mitochondrial matrix (approx. 20%) within the mitochondrial respiratory chain [4]. Superoxide is leaked into the cytoplasm through the mitochondrial permeability transition pore (MPTP) in the outer membrane of the mitochondria [5]. H_2O_2 reacts with superoxide dismutase and inactivates it either in the mitochondrial matrix by MnSod or in the cytosol by Cu/ZnSOD [6, 7, 8].

Generally, the toxic cell oxidants are counteracted and neutralized using several defense strategies which include small reducing molecules maintained at high levels in the cells such as glutathione and ascorbate and several enzymes, such as superoxide dismutase, catalase, and other peroxidases, that further reduce ROS to water [9, 10].

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GSH is an ubiquitous molecule, produced intra-cellularly, with 85-90% freely localized in the cytoplasm. Cytosolic GSH can also be found compartmentalized in several organelles that are subjected to higher oxidative stress, including the mitochondria, the peroxisomes, the nuclear matrix, and the endoplasmic reticulum (ER) [11]. Mammalian cellular machinery maintains homeostatic pools of GSH *via* three different pathways, namely, *de novo* biosynthesis, uptake across plasma membrane of GSH derived from exogenous sources, and NADPH-dependent reduction of oxidized GSH (GSSG) through GSSG reductase-dependent reduction of oxidized GSH (GSSG) through GSSG reductase [12, 13].

Three amino acids, Cysteine, glutamic acid, and glycine, linked linearly by a α-peptide and a α-peptide bond form a tripeptide, glutathione, which is produced mainly by the liver to counteract the redox stress due to cells metabolic machinery [14, 15]. GSH synthesis is catalyzed by enzymes, glutamate cysteine ligase (GCL), and GSH synthetase in an ATP dependent biosynthetic pathway, with cysteine being the rate-limiting substrate [16]. Healthy cells have 90% of glutathione in reduced form, while only 10% is in the form of GSSG. The reduced form purports to be the biologically active form [12]. An increase in oxidative stress leads to a homeostatic increment in the GSSG-to-GSH ratio [17, 18, 19]. The cellular redox state is governed by the concentration and the ratio of NAD^+/NADH, $NADP^+$/NADPH and/or GSH/GSSG. GSH contains thiol (-SH) moiety that acts as reducing agent [20, 21]. Thiol moiety in GSH serves to act as an antioxidant by serving as an electron donor, thereby reducing oxidized proteins in the cytoplasm to cysteine in a process called glutathionylation [21]. In this process, thiol moiety (-SH) gets converted to the oxidized form to give glutathione disulfide (GSSG), also called L-(–)-glutathione [20, 21, 22, 23].

GSH reduces peroxides, catalyzed by glutathione peroxidases (GPx) by serving as an electron donor. H_2O_2 and organic hydroperoxides are metabolized by GPx **Fig. 1** [24, 25]. Since there is no enzyme to reduce GPx, GSH acts reducing agent to GPx, and itself gets oxidized to GSSG. The oxidized glutathione (GSSG) is recycled to its reduced form (GSH), by an enzyme, GSH reductase (GR) using NADPH as an electron donor. Moreover, GSH also acts as a cofactor for oxidized glutaredoxin, formed during the reduction of disulfides [21].

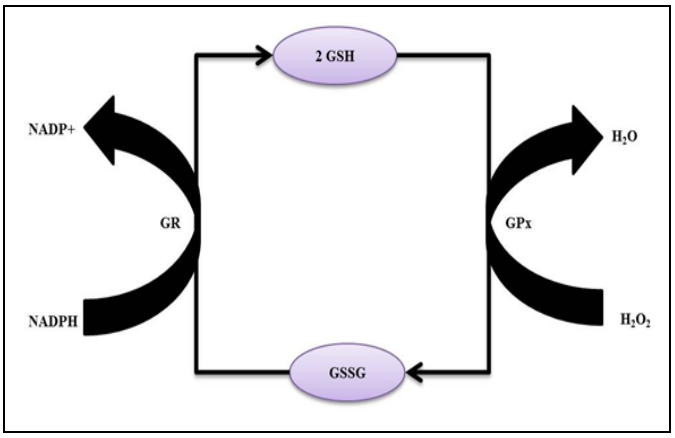

FIG. 1: ROLE OF GSH IN REDUCTION OF PEROXIDES. NADPH ACTS AS AN ELECTRON DONOR

2. Glutathione in Cancer Cell Death: Cancer is the ultimate consequence of the failure of the cellular machinery to multiply normally and response to external or internal insult is overwhelmed such that the mitotic machinery develops aberrations [26].

The development of cancer does have genetic and epigenetic basis [27]. Constant exposure to environmental pollution, dietary insufficiency, radiation, or hazardous chemical exposure along with chronic irritation, infection, or inflammation causes such homeostatic breakdown leading to a growth imbalance, attributable to the mutation of oncogenes and tumor suppressor genes, forfeiting response to cellular death signals. The overall perturbation is an anomalous expression of anti-death and pro-death proteins [28, 29].

The oncogenic stimulation causes an increased cellular metabolism leading to an aberrant increase in ROS stress, which manifolds than that in the normal cells; thus, changing mitochondrial membrane permeability [30, 31]. An increase in free radicals activates cellular defense system for repair or neutralization [32]. However, if the damage is irreversible and depending upon the extent and duration of redox imbalance, the cell progresses towards cell death [33, 34, 35]. However, cancer cells adapt to a survival mode by an increased antioxidant capacity by overexpression of GSH [36] and enzymes responsible for GSH homeostasis, such as GSH peroxidase, GSH reductase, glutaredoxins, and GSH transferases.

Overexpression of GSH has contributed to chemo- and radiotherapy resistance [2, 37].

GSH has shown to afford protection against stress-induced apoptosis [38, 39]. Prevention of apoptosis has shown to be associated with the protection of redox-active catalytic sites at cysteines of the caspases [12]. GSH is necessary for cell survivability as its exhaustion predisposes the cells to free radical attack [40]. Various basic cellular components like lipid, protein, carbohydrate, and DNA are susceptible to attack by free radicals [41]. An increase in cellular oxidative stress causes deleterious derangements supporting cell death such as single- and double-strand DNA fragmentation, damages in mitochondria causing permeability alterations by decreasing the transmembrane potential, and promoting the extrusion of death-related molecular signals [37, 42]. Therefore, inhibiting GSH synthesis by an agent such as L-buthionine-(S, R)-sulfoximine (BSO) causes GSH depletion [43] and hence, can act as a potential target to sensitize resistant tumor cells [44] **Fig. 2**. Simon *et al.*, have also shown that BSO tends to sensitize cancer cells to chemotherapeutic agents [45].

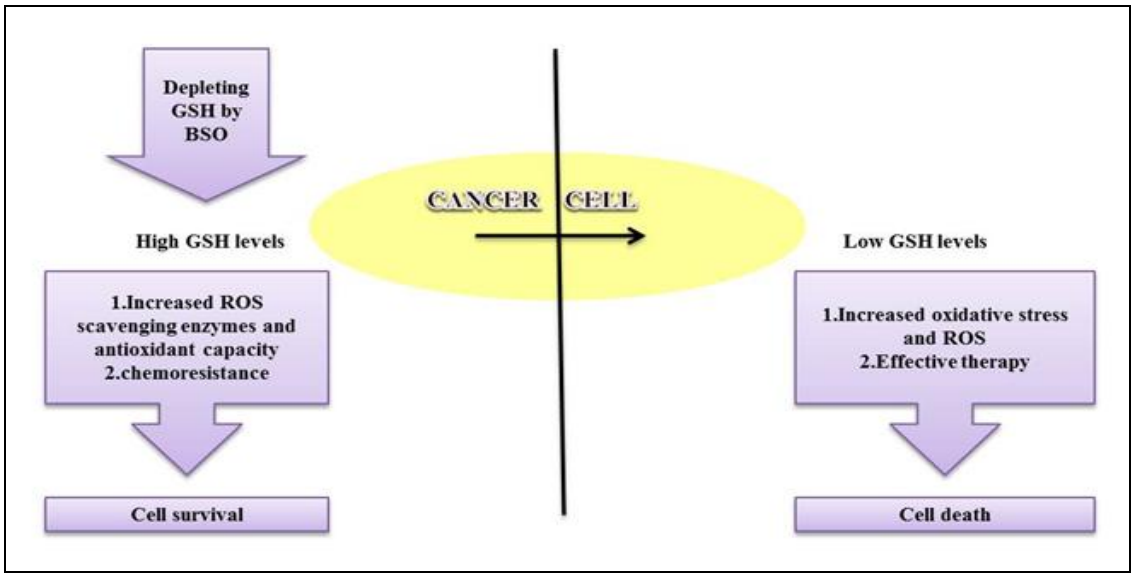

FIG. 2: EFFECT OF CELLULAR GSH DEPLETION IN CANCER CELLS: HIGH CELLULAR GSH LEVELS ARE A SURVIVAL ADAPTION OF CANCER CELLS TO ACCOMMODATE HIGH METABOLIC STRESSORS SUCH AS ROS, MAKING THEM RESISTANT TO CHEMOTHERAPY. HOWEVER, CELLULAR DEPLETION OF GSH RENDERS CELLS MORE VULNERABLE TO ROS ATTACKS

Apoptosis due to the oxidative stress can be activated either through an intrinsic pathway (mitochondria-derived) or extrinsic pathway (death receptor-mediated) former involving procaspase-9 activation while the latter involves activation of membrane receptors without activation of mitochondrial pro-apoptotic events [46]. Furthermore, GSH depletion substantially increases ROS, thus activating autophagy [47]. Increased ROS leaves the cell prone to oxidative damage, which in course if exceeds repairability of the cellular machinery, calls for activation of autophagy involving the formation of autophagosome that digests organelles [48]. And if the damage is irreversible, autophagy becomes a pro-death mechanism [49].

3. GSH Depletion and Mechanisms of Cancer Cell Death: Cancer cells are immortal due to alterations in pathways that normally maintain the equilibrium between cell survivability and cell senescence [50]. Any alteration in cell death can lead to multiple pathological diseases like cancer, aging, neurodegenerative disorders, cystic fibrosis [51].

Manipulating intracellular GSH by drugs such as BSO (inhibition of GSH synthesis) [52], Cys starvation (required for GSH synthesis) or γ-glutamate-cysteine ligase knockdown (in cultured cells) have shown to induce cell death, with or without inducing apoptosis [53, 54, 55].

Moreover, cytosolic efflux of GSH through MRP1 [56], direct GSH oxidation by ROS or hindering GSH transport across mitochondria facilitates permeability transition pore opening, thereby activating mitochondrion-based death mechanism[14] **Fig. 3**.

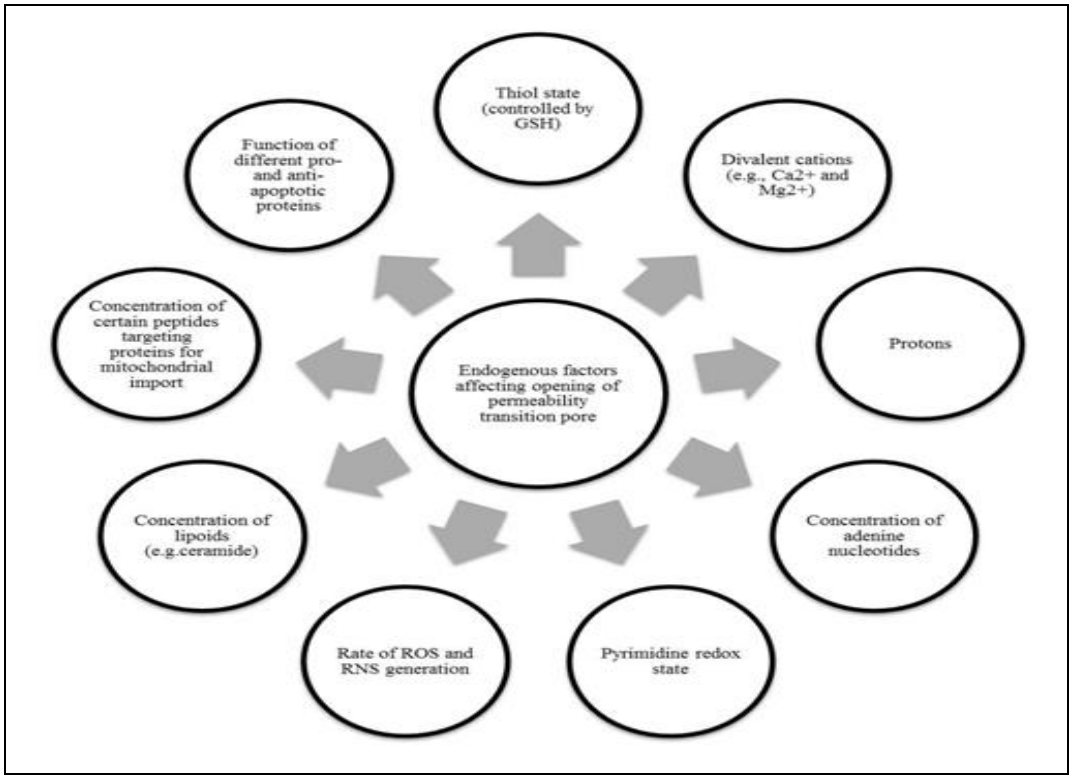

FIG. 3: ENDOGENOUS FACTORS AFFECTING OPENING OF PERMEABILITY TRANSITION PORE

GSH has found to exert most of its actions *via* modulating gene transcription [58]. The effects of GSH are regulated by various transcription factors. For instance, redox-sensitive transcription factor, NF-E2 p45-related factor-2 (Nrf2) regulates GSH-related enzyme activities, which is itself under GSH regulation [59]. Nuclear factor erythroid 2–related factor 2 (Nrf2), a basicleucine zipper (bZIP) protein regulates the basal and induced expression of antioxidant response element-dependent genes. Nrf2 binds to antioxidant response elements (ARE) and stimulates transcription of detoxification-related genes [60]. Oxidation of thiol-containing proteins induces the release of Nrf-2, which thereafter translocate to the nucleus. Association of Nrf-2 to antioxidant response elements (ARE) in the control regions of multiple detoxification-related genes, activates gene transcriptions [61].

In the event of GSH depletion caused by BSO, Nrf2 is unregulated to provide antioxidant defense. Several studies demonstrate that Nrf-2-deficient murine embryonic fibroblasts lack this defense capacity leading to concentration, caspase-3 activation, and cellular death. Furthermore, Nrf-2 deficient cells have been found to be highly susceptible to doxorubicin and BSO treatment-induced cell death than wild cells [62]. Furthermore, events leading to oxidation or reduction of critical Cys residues in the DNA binding domain of several transcription factors affect interactions with specific DNA bases [63]. Oxidation of these Cys residues alters the 3D structure of transcription factors, which in turn affects its function [64, 65]. Functional changes in any of these transcription factors affect gene expression of NF-κB, p53, MAPK, *etc*. by either upregulating or downregulating the process. Several MAPKs, including ERK, JNK, and p38 are known to have a crucial role in stress-induced apoptosis [66, 67, 68].

3.1 GSH and Apoptosis: Apoptosis or programmed cell death type 1 is a highly organized mechanism of controlled cell death, induced by diverse form of stimuli leading to terminal activation of cysteine-aspartate proteases [69]. Various precise non-inflammatory apoptotic pathways undergo progressive activation, leading to specific bio-chemical and morphological cellular aberrations. Deregulation of apoptosis is found to be either a cause or consequence in several pathological conditions including cancer, auto-immune disorders, and neurodegeneration [70, 71].

Cytotoxic agents, such as chemotherapeutics, xenobiotics, and metals produce oxidative stress by

inducing GSH depletion, which eventually lead to apoptosis [72, 73]. This may be attributable to either oxidation of GSH to GSSG by reactive oxygen/nitrogen species or due to conjugation with highly reactive compounds. In contrast, apoptosis induced by stimuli other than reactive species, such as activation of death receptors, is found to be mediated by an efflux transport of GSH through plasma membrane [74]. GSH is crucial for cell survival. Its depletion or extrusion renders the cell susceptible to death-receptor activation or mitochondrial apoptotic signaling making this strategy useful in rendering chemo- and radio-therapy resistant cells vulnerable to cell death [75, 76] **Fig. 4**.

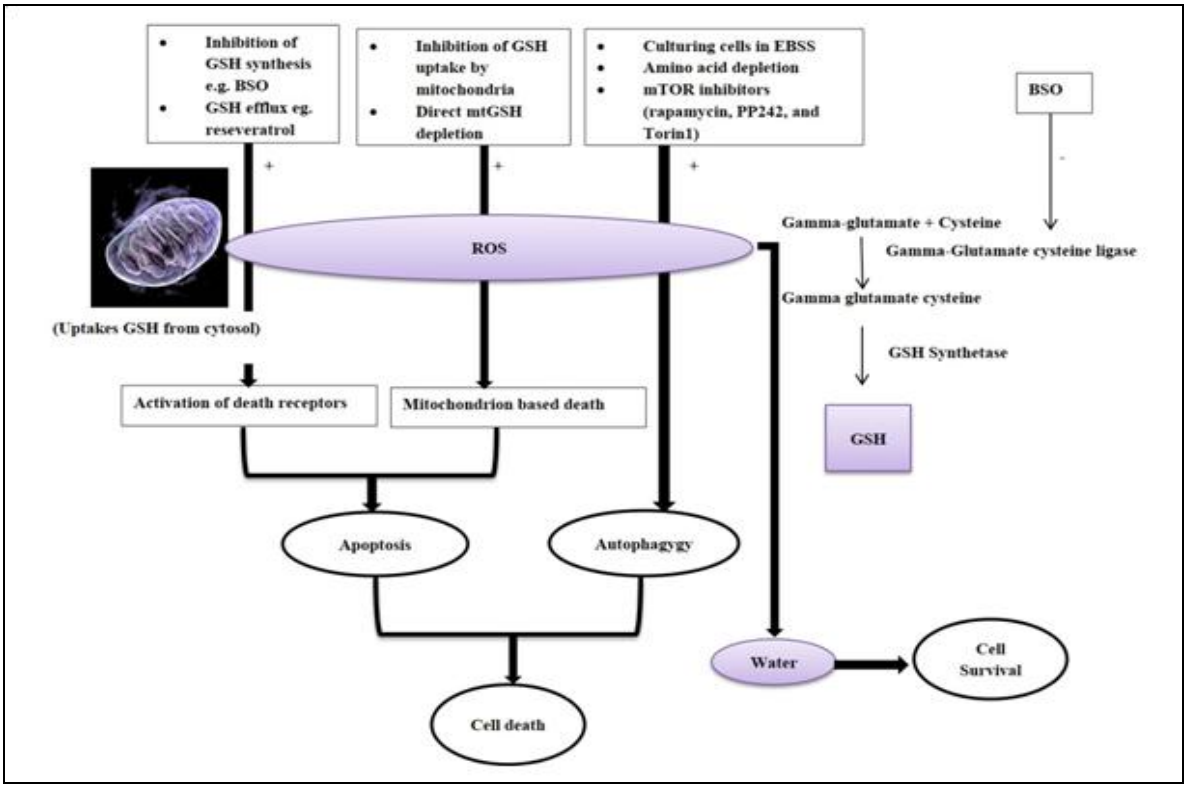

FIG. 4: ROLE OF GSH MODULATION IN VARIOUS PATHWAYS OF CELL DEATH

BSO selectively inhibits γ-glutamate-cysteine ligase, depleting GSH without triggering apoptosis but facilitating other death-related mechanisms. For example, BSO potentiates death-receptor-induced apoptosis in cells [77]. An increase in cellular ROS/RNS leads to loss of mitochondrial integrity, thereby activating the intrinsic apoptotic pathway [78, 79].

Permeability transition pore at the inner-outer membrane contact of mitochondria is voltage- and Ca^{2+}-dependent, cyclosporine A-sensitive [80, 81], high conductance channel whose permeability suddenly increases for water and solutes with molecular masses of up to 1,500 Da [12]. Compromised mitochodrial integrity is followed by an increased osmosis leading to swelling of mitochondria, rupture of the outer mitochondrial membrane, and release of pro-apoptogenic proteins into the cytosol [82]. Pro-apoptotic factors released include apoptosis-inducing factor (AIF) [83] or second mitochondria-derived activator of caspases / direct IAP [84]. Cyt c released from mitochondria binds to Apaf-1 (apoptotic protease-activating factor-1), forming apoptosome which recruits procaspase-9 [84]. Downstream cleavage/activation of effector caspases, -3 and -6/7 is signaled by ATP-dependent scission of procaspase-9 [85].

Furthermore, GSH efflux by compounds such as resveratrol, a plant polyphenol induces experimental apoptosis by a mechanism involving BAX overexpression-mediated apoptosis, which is a ROS independent mechanism [28, 49, 86]. ROS, electrophiles, and phenolic oxidants induce Nrf2 transcription factor to activate several detoxification-related genes including those for γ-glutamate-cysteine ligase [28].

Genetically altered mice deficient in Nrf-2 (Nrf-$2^{-/-}$) are found to be vulnerable to the damaging effects of hyperoxia, as indicated by an increase in pulmonary perme-ability, macrophage infiltration and epithelial injury in comparison to control wild mice [87, 88]. Cell apoptosis is also found to be regulated by GSH/ GSSG redox status [17]. According to one study, apoptosis was found to be regulated by a consistent GSH/GSSG imbalance, whereby a rise in GSSG produces kinetically accelerated loss of mitochondrial integrity, translocation of Cyt c from mitochondria-to-cytosol followed by caspase-3 activation [89]. A significant rise in GSSG was found to occur within a narrow window of redox shift [90]. During an initial 30 min post-oxidative challenge, followed by cellular GSH/GSSG balance recovery by 1 h had no influence on the apoptotic endpoint, a response that is coherent with early initiation of redox signaling. Pretreatment with a thiol antioxidant, N-acetylcysteine (NAC) could block oxidant-induced cell apoptosis by preventing an increase in GSSG [91]. Howsoever, NAC does not prevent the oxidative stress once the rise in GSSG has occurred [92].

Mitochondria do not synthesize GSH but uptakes it from cytosol through a multicomponent transport system [14]. The influxed GSH is the source of antioxidant defense against peroxides generated *via* ETC; regulating mitochondrial permeability transition and permeability transition pore opening [93]. Mitochondrion-based cell death can be made feasible by either targeting GSH uptake of mitochondrion or through direct mtGSH depletion. This could be an attractive strategy to sensitize malignant cells to molecular effectors (*e.g.*, oxidative stress inducers and/or cytotoxic drugs) and cause mitochondrion targeted cell death [94].

3.2 GSH and Autophagy: Autophagy or programmed cell death type II is a natural, regulated, destructive mechanism of the cell whereby the targeted cytoplasmic organelles are engulfed within lysosome to form the double-membrane vesicle, autophagosome [95]. The autophagosome eventually fuses with lysosomes to form autolysosome and the contents are degraded by different acid hydrolases and recycled [96]. While autophagy is a mechanism to digest cellular debris, worn out organelles and intracellular pathogenic organisms, if the stimuli are persistent, it becomes a pro-death mechanism [97, 98]. Lysosomes are home to more than 50 soluble acid hydrolases that are meant to perform a cellular digestive function and over 120 lysosomal membrane proteins to maintain the structural integrity of the organelle, regulate lysosomal trafficking, fusion, and intralysosomal pH. The intra-organelle pH is highly acidic *i.e.* pH 4.5-5.0 which is crucial for the optimal catalytic activity of its hydrolytic enzymes [99, 100]. Defective autophagic machinery compromises cellular recycling mechanism and leads to several pathological conditions such as cancer and neurodegenerative diseases [101, 102]. Several stimuli can induce autophagy experimentally. This includes starvation (by culturing cells in Earle's Balanced Salt Solution (EBSS)), ROS, amino acid deprivation, and three different mTOR inhibitors, rapamycin, PP242 and Torin1 [103, 104]. ROS is crucial for inducing starvation-induced autophagy. As demonstrated by Scherz-Shouval *et al.,* [105] starvation-induced autophagy targets ATG4, a thiol-containing protein that initiates early step of autophagosome formation [106].

A relation between starvation and GSH levels were elucidated in a study carried out by Desideri *et al*., whereby, cervix carcinoma HeLa cells were cultured in HBSS (to mimic nutrient starvation) and the GSH levels were analyzed by HPLC technique. Results concluded a time-dependent decrement in GSH concentration from 3 h of nutrient removal. Moreover, a progressive decrement in GSH/GSSG ratio was observed owing to decreasing GSH levels but a non-significant change in GSSG levels. Nutrient starvation in HeLa cells, as well as in HepG2 and H1299 cells, has shown to produce a significant increase in extracellular GSH levels post 3h nutrient starvation. And observed GSH efflux was not due to cells undergoing apoptosis 66 as there was no significant activation of markers for caspase-dependent apoptosis *i.e.* caspase 9/3 (CASP9/3), 24 h prior to starvation in HeLa and H1299 cells and 48 h in HepG2 cell, further indicating that a reduction/extrusion of GSH was not a consequence of cells undergoing apoptosis [95, 96].

Autophagy protein 5 (ATG5) and its complex in ATG12-ATG5:ATG16L causes elongation of the phagophore in the autophagic pathway [105]. Studies including a knockdown model of ATG5 were not

found to counteract the decrease in GSH, showing this isn't brought about *via* autophagy initiation [98]. As GSH is a ubiquitous molecule, its role in autophagy is not completely elucidated, but several studies demonstrate that N-acetyl-L-cysteine induced replenishment of cellular GSH halters autophagy induction along with autophagosome formation and protein degradation induced by starvation [107, 108].

There has been a correlation between intracellular GSH pools and induction of mitochondrial autophagy (mitophagy) as studied in yeast by Deffieu et al. In the study by Deffieu *et al.*, involving regulation of mitophagy in yeast, 3h after nitrogen starvation, 75% of the cells show the vacuolization of mitochondria. However, in the presence of NAC, 13% of the cells had their mitochondria/vacuoles in contact after 2 h of starvation, while 13% of the cells exhibited vacuolar sequestration of mitochondria after 3 h of starvation. The observations were a conclusive indication of the protective effect of NAC, as shown by impaired mitochondria/vacuoles contacts and mitochondria sequestration in vacuoles. The study though established the protective role of NAC against nitrogen starvation-induced mitophagy but the correlation between GSH thiol redox state and the induction/execution of autophagy in mammalian cells, is insufficient [109].

CONCLUSION: GSH is crucial for the survival of cells against a battle with free radicals generated as a byproduct of cellular metabolism or at an encounter with xenobiotics, ionizing radiations, and oxidative stress-inducing biotherapy. Tumor cells have the advantage of producing a high level of intracellular GSH and hence, high antioxidant capacity, therefore, a novel strategy to selectively deplete GSH in cancer cells offers a promising treatment approach in drug and radiation-resistant cases. For instance, GSH concentration in mammary gland tumors range between 10–40 nmol/mg protein, while for disease-free breast tissue, this value falls down to a range between 1–10 nmol/mg-protein [110]. The heterogeneous nature of tumor cells provides them with an excellent armor to counteract the death-inducing stimuli, where different cell subset show different states of resistance where one strategy may include overproduction of GSH. Depletion of cytosolic or mitochondrial GSH by promoting efflux or inhibiting uptake predisposes cells to oxidative stress, thereby facilitating the release of death-inducing molecular signals ending in apoptosis or autophagy.

BSO inhibiting γ-glutamate-cysteine ligase (depleting GSH without triggering apoptosis) or promoting GSH efflux by compound like resveratrol (triggering apoptosis) [110] or inducing autophagy through several stimuli like starvation (by culturing cells in Earle's Balanced Salt Solution (EBSS)), ROS, amino acid deprivation and three different mTOR inhibitors, rapamycin, PP242, and Torin1 [111, 112] appears to be promising but considering the heterogeneous nature of tumors and non-uniform levels of GSH in different cell types, ways to selectively deplete it from the cancer cells without jeopardizing the normal healthy cells is challenging.

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CONFLICTS OF INTEREST: None

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