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REVIEW / DERLEME

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Assessment of Ferroptotic Cell Death and Related Treatment Targets in Neuroblastoma

Nöroblastomda Ferroptotik Hücre Ölümü ve İlişkili Tedavi Hedeflerinin Değerlendirilmesi

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Abstract

Ferroptosis is defined as an iron-dependent, non-apoptotic programed cell death modality that occurs due to an imbalance of intracellular redox hemostasis. Recently, ferroptosis has attracted attention in cancer research and has been shown to play a role in numerous oncogenic pathways. Studies have revealed that increased levels of intracellular reactive oxygen species play critical roles in oncogenic processes such as tumorigenesis, angiogenesis, invasion, metastasis, and chemoresistance because of their role in ferroptotic cell death. Neuroblastoma is the most common extracranial solid tumor in children and represents 8-10% of all pediatric cancers and 1/3 of all malign diseases of infancy. As seen in all types of cancers, the development of chemoresistance seriously affects the success of neuroblastoma treatment. Tolerance to chemotherapy in neuroblastoma was associated with the induction of exogenous defense genes and reduction of ferroptosis susceptibility biomarkers. Therefore, ferroptosis is a potential druggable driver in cancer treatment. In this review, studies associated with ferroptosis and neuroblastoma to date were reviewed and literature data were assessed in terms of ferroptotic mechanisms in neuroblastoma and potential treatment targets.

Keywords: Ferroptosis, neuroblastoma, treatment targets

Öz

Ferroptoz, hücre içi redoks hemostazındaki dengesizlik nedeniyle ortaya çıkan, demire bağımlı, apoptotik olmayan, programlanmış bir hücre ölümü şekli olarak tanımlanır. Son zamanlarda ferroptoz kanser araştırmalarında dikkat çekmiş ve birçok onkojenik yolda rol oynadığı gösterilmiştir. Çalışmalar, artan hücre içi reaktif oksijen türlerinin seviyelerinin, ferroptotik hücre ölümündeki rolü nedeniyle tümör oluşumu, anjiyogenez, invazyon, metastaz ve kemorezistans gibi onkojenik süreçlerde kritik rollere sahip olduğunu ortaya çıkarmıştır. Nöroblastom çocuklarda en sık görülen ekstrakraniyal solid tümördür ve tüm pediyatrik kanserlerin %8-10'unu, bebeklik çağının malign hastalıklarının ise 1/3'ünü oluşturur. Tüm kanser türlerinde görüldüğü gibi nöroblastomda da kemorezistansın gelişmesi tedavi başarısını ciddi şekilde etkilemektedir. Nöroblastomda kemoterapiye toleransın, ekzojen savunma genlerinin indüksiyonu ve ferroptoz duyarlılığı biyobelirteçlerinin azalmasıyla ilişkili olduğu belirtilmiştir. Bu nedenle, ferroptozun kanser tedavisinde ilaçla hedeflenebilir bir etken olması muhtemeldir. Bu derlemede ferroptoz ve nöroblastom ile ilgili bugüne kadar yapılan çalışmalar gözden geçirilmiş ve literatür verileri nöroblastomdaki ferroptotik mekanizmalar ve potansiyel tedavi hedefleri açısından değerlendirilmiştir.

Anahtar Kelimeler: Ferroptoz, nöroblastom, tedavi hedefleri

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Introduction

Ferroptosis is defined as an iron-dependent, non-apoptotic programed cell death modality. Ferroptosis occurs when the cellular levels of lipid reactive oxygen species (ROS) outweigh the glutathione peroxidase (GPX4) activity. As a result, cellular redox hemostasis is disrupted and cell death occurs[1]. Neuroblastoma (NB) is the most common extracranial solid tumor in children. The aim of this review was to address the mechanisms of action of ferroptosis suggested so far and to discuss ferroptosis-associated potential treatment targets in NB.

Molecular Mechanism of Ferroptosis

The fingerprint characteristic of ferroptosis is the generation of ROS, mostly due to an imbalance in iron metabolism[2]. Circulated iron (Fe^{3+}) uptake is processed by its attachment to transferrin (TF) and the transferrin receptor (TFR1). Iron Fe^{2+} is formed by the deoxidation of Fe^{3+} by a reaction catalyzed by the six-transmembrane epithelial antigen of prostate 3 (STEAP 3). Iron Fe^{2+} is readily soluble and has a high electron transfer capacity; therefore, it is taken up to the labile iron pool (LIP). The LIP repertoire comprises circulated iron uptake and ferritinophagy (ferritin degradation). Excessive LIP formation may initiate the Fenton reaction, which generates ROS because of the interaction between hydrogen peroxide (H_2O_2) and iron Fe^{2+}[3]. Research has suggested that iron overload resulting from increased iron intake and/or reduced iron storage finally leads to ferroptosis. This was demonstrated in a study in which ferroptosis-sensitive cells with RAS mutation showed increased TFR1 expression and decreased ferritin light and heavy chain 1 (FTL-FTH1) expression compared with ferroptosis-insensitive cells without RAS mutation[4].

Inhibition of antiporter system Xc^- or inactivation of enzyme GPX4 are responsible for ferroptosis initiation. System Xc^- mediates the importation of extracellular cystine (Cys2) accompanied by the exportation of intracellular glutamic acid (Glu)[5]. These amino acids (Cys2 and Glu) together with glycine (Gly) are essential for the generation of the major intracellular antioxidant glutathione (GSH), which reacts with the enzyme GPX. Intracellular cysteine levels are also increased by the transsulfuration of methionine (Met). Another mechanism contributing to cysteine levels in cells is the transporter system alanine/serine/cysteine (ASC), which mediates cysteine uptake[3]. Enzymes such as lysophosphatidylcholine acyltransferase 3 (LPCAT3), acyl-CoA synthetase long-chain family member 4 (ACSL4), and lipoxygenase (LOXs) mediate the reaction chain of phosphatidylethanolamine [(PE)-PUFAs-OOH] formation from free polyunsaturated fatty acids (PUFAs) via peroxidation[6].

Under physiological conditions, membrane lipid metabolism is mediated by the enzyme GPX4 and GSH availability. A recent study suggested that the breakdown of membrane lipids may be mediated by the key reductase GPX4[7]. The ferroptosis-initiating step is the inactivation of GPX4, followed by the importation of Iron (Fe) via TRF1. Subsequently, divalent metal ion transporter 1 transfers the ferrous ions to the cytosol. However, under ferroptotic conditions, membrane lipids are oxidized to Lipid-ROS (L-ROS) by the Fenton reaction directly bypassing the GPX4 pathway. The Fenton reaction, which is carried out with electrons from ferrous ions, is induced by cysteine deprivation or excessive numbers of intracellular ferrous ions. The Fenton reaction is one of the major pathways of ferroptosis. The Fenton reaction is responsible for cellular damage by oxidation of cellular substrates by hydrogen peroxide and iron. Low oxygen levels trigger L-ROS to attack vital intracellular molecules, especially DNA and RNA, resulting in imbalanced cellular homeostasis, and finally, cellular death occurs (Figure 1)[8].

Ferroptosis and Cancer

Ferroptosis was first discovered in RAS-expressing cancer cells with a small molecule called erastin, which had a lethal effect in an iron-dependent manner, unlike other cell death modalities described before. Ras-selective small molecule 3 (RSL3) was found to induce this type of cell death, and these small molecules were defined as ferroptosis-inducing agents. Following these findings, the research groups focused on the relationship between ferroptosis and Ras oncoprotein. It was observed that cell lines with WT Ras oncoprotein (fibrosarcoma and kidney tubule cells) were sensitive to erastin, whereas cells with Ras mutation (rhabdomyosarcoma cells) were resistant to RSL3 and erastin[9].

Studies have suggested that ferroptosis may represent an adaptive mechanism essential for the eradication of malignant cells. This phenomenon was clarified by studies on the well-known tumor suppressor protein p53 (TP53). Mutated $TP53^{3KR}$ was no longer capable of inducing apoptosis, senescence, and cell cycle arrest, thus losing the ability to inhibit malignant transformation. However, $TP53^{3KR}$ could still induce ferroptosis, which is promising for tumorigenesis inhibition (Figure 2)[10].

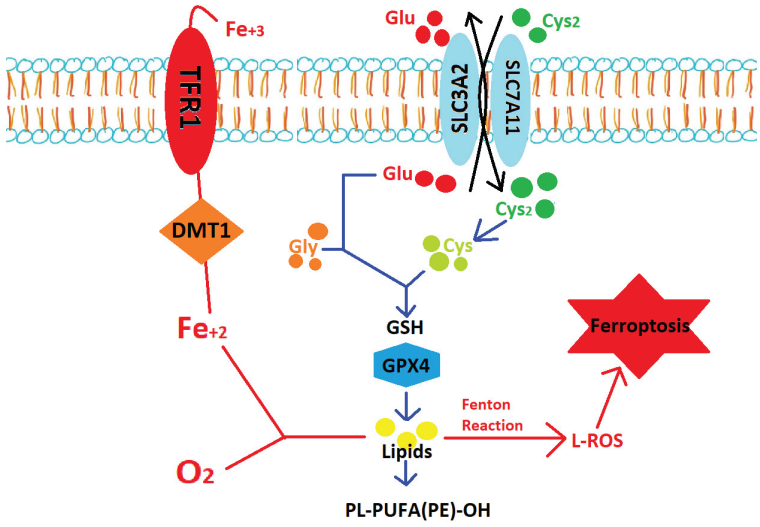

Figure 1. Normal cellular lipid metabolism and mechanism of ferroptosis. Under physiological conditions, membrane lipid metabolism is mediated by the enzyme GPX4 and the availability of GSH (blue arrows). However, under ferroptotic conditions, membrane lipids are oxidized to L-ROS by Fenton reaction directly bypassing the GPX4 pathway and as a result, ferroptosis occurs (red arrows)

Cys: Cysteine, Cys2: Cystine, DMT1: Divalent metal (Ion) transporter 1, Glu: Glutamine, Gly: Glycine, GPX4: Glutathione peroxidase 4, GSH: Glutathione, JNK: SLC7A11: Solute carrier family 7 member 11, SLC7A11: solute carrier family 3 member 2, L-ROS: Şipid-reactive oxygen species, PL-PUFA-OH: Phospholipid polyunsaturated fatty acid alcohols, TFR1: Transferrin receptor 1

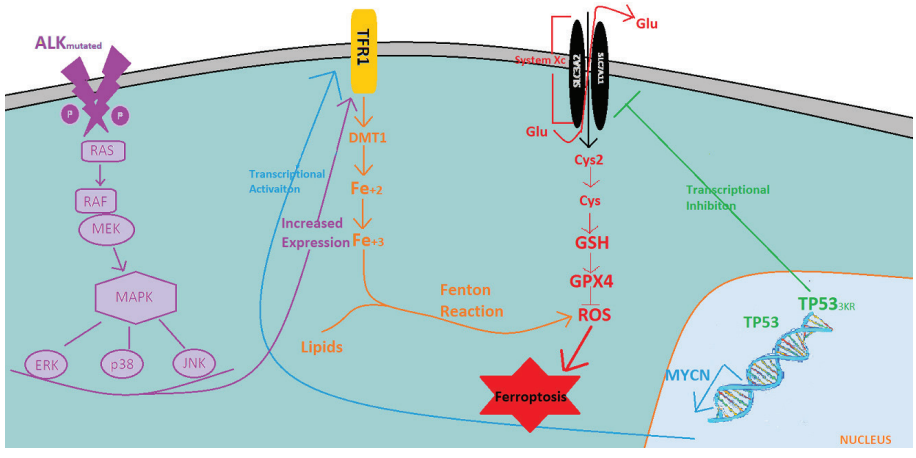

Figure 2. Involved mechanisms in neuroblastoma and their association with ferroptosis. Amplified MYCN in NB causes intracellular higher levels of iron and ROS accumulation via increasing TFR1 expression by transcriptional activation (blue arrows). Mutated TP53 (TP533KR) in NB supports ROS generation by blocking GPX4 enzyme activity by limiting GSH synthesis via the inhibition of SLC7A11 expression and therefore limiting the import of cystine through system Xc antiporter (green arrows). RAS-RAF-MAPK pathway is one of the downstream pathways of ALK in neuroblastoma. MAPK family members, ERK, p38, and JNK, cause ferroptosis by increasing TFR1 expression in RAS mutated NB cells (purple arrows)

ALK: Anaplastic lymphoma kinase, Cys: Cysteine, Cys2: Cystine, DMT1: Divalent metal (Ion) transporter 1, ERK: Extracellular signal-regulated kinases, Glu: Glutamine, GPX4: Glutathione peroxidase 4, GSH: glutathione; JNK: C-jun n-terminal kinase, MAPK: Mitogen-activated protein kinase, MEK: Mitogen-activated ERK-activating kinase, NB: Neuroblastoma, RAF: Rapidly accelerated fibrosarcoma; RAS: Rat sarcoma virus, SLC7A11: Solute carrier family 7 member 11, ROS: Reactive oxygen species, TFR1: Transferrin receptor 1, TP53: Tumor protein 53

The vulnerability of cancer cells to ferroptosis may be due to the activation of the Ras-MEK (mitogen-activated protein kinase) signaling pathway because the Ras-MEK pathway promotes iron excess in malignant cells by regulating the levels of TRF1 and ferritin expression. Upregulation of the Ras-MEK pathway can advance ROS generation by inhibiting cellular cysteine import or voltage-dependent anion channel 2/3 (VDAC 2/3), thus sensitizing cancer cells to ferroptosis[11].

Recently, ferroptosis has attracted attention in cancer research and has been shown to play a role in numerous oncogenic pathways. Ferroptosis has been suggested to be a target in processes such as tumorigenesis, angiogenesis, invasion, and metastasis. In addition, it was hypothesized that ferroptosis can contribute to combat chemoresistance and increase the effectiveness of cancer immunotherapy[12,13].

Based on the strong relationship between ROS and cell death, strategies that increase ROS generation or downregulate oxidative defense mechanisms have become the main focus of cancer treatment research. These strategies were strengthened by the study of Galadari et al.[12], which revealed that cancer cells have higher levels of ROS than healthy cells. High levels of intracellular ROS catalyze tumorigenesis by damaging or modifying cellular proteins, DNA, and lipids[14]; support angiogenesis by modifying vascular endothelial growth factors or by regulating tubular formation, migration, and proliferation[15]; contribute to invasion and metastasis by modulating signal cascades and the cellular skeleton[16]; and play a role in chemoresistance[17]. Ferroptotic cell death becomes prominent because of the same strong relationship in all these tumor-promoting cellular processes that occur because of high levels of ROS in cancer cells. Therefore, two different approaches have emerged to target cancer treatment options; one of these is to decrease cellular ROS levels and the other is to increase intracellular ROS levels to a toxic state and trigger ferroptosis[18].

NB is the most common extracranial solid tumor in children and represents 8-10% of all pediatric cancers and 1/3 of all malign diseases of infancy. The overall 5-year survival rate in low- and moderate-risk groups is over 90%, whereas it is lower than 50% in the high-risk group, which represents approximately half of all patients[19].

The most common genetic and epigenetic changes in NB are the expression alterations of MYCN, ALK (anaplastic lymphoma kinase), *PHOX2B* (paired-like homeobox 2b), *ATRX* (alpha-thalassemia/mental retardation, X-linked), *TERT* (telomerase reverse transcriptase), TP53, Histone deacetylase (*HDAC*), Lysine methyltransferase (*KMTs*), and histone lysine demethylase (*KDM*) genes[20]. Among these, MYCN amplification and 17q chromosome gain are the most well-known. In addition, 1p and 11q chromosome deletions and hyperploidy are frequently detected[21]. MYCN amplification and 1p and 11q deletions are related to poor prognosis, whereas hyperploidy is associated with a favorable prognosis[22]. In addition, ALK was defined as an oncogene associated with familial and sporadic NB[23].

As seen in all types of cancers, the development of chemoresistance seriously affects the success of treatment in NB. O-6-methylguanine-DNA-methyltransferase, which is a DNA methyltransferase that interacts with the Wnt/B-catenin signaling pathway, is upregulated in NB and is associated with chemoresistance[24]. Increased levels of HDAC8 (histone deacetylase 8) in NB cells were proposed to contribute to chemoresistance by suppressing the expression of miR-137 and triggering the expression of the multidrug resistance protein 1 (*MDR1*) gene[25]. MiR-17-92 cluster members are upregulated in NB cells and patients with MYCN amplification by the regulation of p21 (a cyclin-dependent kinase inhibitor 1A, a cell cycle regulator) and BIM (bcl-2-like protein 11, an apoptotic regulator)[26]. MYCN plays a critical role in resistance to platin-based molecules by inhibiting apoptosis via deregulating PPARG coactivator 1 alpha (*PPARGC1A*) and mitochondrial transcription factor A (*TFAM*) genes[27].

To overcome chemoresistance in NB, it is necessary to focus on other cell death modalities. Recent studies have shown that stimulation of ferroptosis in cancer cells can be a novel cancer treatment strategy[28] and have aimed to accelerate the clinical application of ferroptosis targeting[29]. To date, various strategies have been developed to induce ferroptosis in NB[30]. The aim of this review is to provide an overview of the ferroptotic mechanisms in NB and potential treatment approaches that can be developed via these mechanisms.

Method

In this review, all data present in "PubMed" database between 2002 and 2021 years were assessed and analyzed in terms of ferroptosis and related treatment approaches. A comprehensive search of peer-reviewed journals but no conference papers or reports was completed based on a wide range of keywords such as "ferroptosis", "GPX4", "GSH" and "NB". Original research articles assessing the role of ferroptosis in NB were reviewed and included in this review.

Results

Studies on the modulation of ferroptotic machinery in NB are summarized in Table 1 and detailed explanations are given below.

Buthionine sulphoximine (BSO) was identified as a glutathione synthesis inhibitor that sensitizes NB cells to melphalan by inducing ferroptotoic cell death. In this study, a panel of 20 different NB cell lines was tested. Most of these cell lines, including post-autologous hematopoietic stem cell transplantation cell lines, which are severely resistant to myeloablative melphalan levels and lack p53 function, became sensitive to clinically achievable levels of melphalan and BSO when combined[31].

Overexpression of mitochondrial ferritin in SHSY-SY NB cells increased the cells' resistance to oxidative stress and protected them from ferroptosis[32]. In a transgenic *drosophila* NB model, overexpression of mitochondrial ferritin suppressed erastin-induced ferroptosis[33].

Silencing of the iron export protein; FPN in SH-SY5Y human NB cells accelerated erastin-induced ferroptosis by increasing lipid ROS accumulation[34]. Therefore, ferroportin inhibitors can be used as chemosensitizer in neuroblastoma. Similarly, in another study, HDAC inhibitors were identified as a new class of chemotherapeutics because they minimize neuronal toxicity and contribute to tumor suppression by inducing ferroptosis[35].

A different study performed with SHSY-5Y NB cells reported that isoflurane triggers ferroptosis via the inhibition of cystine/glutamate antiporter activity by the formation of the Beclin1-Solute Carrier Family 7 Member 11 (SLC7A11) complex[36].

Ferroptosis has also been reported to be effective in refractory, high-risk NB. Withaferin-A (WA) was shown to be effective in NB by inducing both canonical and non-canonical ferroptotic pathways. On the one hand, WA induced the canonical ferroptotic pathway by reducing GPX4 protein levels and activity. On the other hand, WA induced a non-canonical ferroptotic pathway by increasing the labile Fe (II) pool via overactivation of heme oxygenase 1 (HMOX1) as a result of direct targeting of kelch-like ECH-related protein 1 (KEAP1). This bidirectional mechanism of WA, when compared with etoposide and cisplatin, was shown to be significantly more effective in killing a heterogeneous panel of high-risk NB cell lines and in reducing tumor growth and relapse in NB xenografts[37]. At the same time, Withaferin a nanoparticles (NP) were engineered and these NPs were reported to decrease tumor growth because they caused a better accumulation of the molecule in the tumor site via nanotargeting by systemic administration[37].

MYCN amplification constitutes a 20-25% portion of NB cases and a major percentage of pediatric cancer-related deaths. Amplified MYCN remodels the cell by the expression of key receptors and increases iron influx by the increased expression of TFRC1 (Figure 2). Accumulated iron causes ROS formation, and MYCN-amplified NB cells become more dependent on the Xc-cysteine/glutamate antiporter system for ROS detoxification. This dependency causes significant sensitivity to the targeting of the Xc-cystine/GSH pathway by ferroptosis inducers. Therefore, agents that target GPX4 or TFRC are potential strategies for treating MYCN-amplified NB[38]. FDA-approved molecules for rheumatoid arthritis, sulphasalazine, and auranofin can be tested for NB treatment. In MYCN-amplified patient-derived xenograft models, these two molecules stopped tumor growth and induced ferroptosis[39]. In another study performed with sulphasalazine, cancer stem cells (CSCs) were isolated from both etoposide-resistant and etoposide-sensitive NB cells, and CSCs were treated with etoposide alone or in combination with sulphasalazine or C2-4 (a PKC-α inhibitor). The combination of etoposide with sulfasalazine or C2-4 prevents the spread of cancer stem cells by avoiding epithelial-mesenchymal transition (EMT) and decreasing intracellular GSH levels. The results of this study indicate that these effects are caused by the downregulation of GPX4 and the triggering of ferroptosis by lipid peroxidation[40]. In another study, sulphasalazine was applied to a panel of MYCN-amplified and non-amplified NB cell lines, and it was shown that sulphasalazine exerts anti-tumor effects by triggering ferroptotic cell death rather than apoptosis[41].

Another study reported that MYCN sensitizes NB cells to ferroptosis when the intracellular cysteine availability required for glutathione synthesis is limited. A high MYCN state in NB cells causes lipid peroxidation and triggers ferroptosis via an acute intracellular cysteine decrease. These results can explain the spontaneous regression observed in NB patients[42].

Conclusion

Agents that inhibit GPX4 and GSH-mediated detoxification directly or indirectly and cause an increase in intracellular iron accumulation are potential treatment options for ferroptotic cell death in NB. These findings indicate that targeting ferroptosis in treatment-resistant or MYCN-

Table 1. Studies on the modulation of ferroptotic machinery in neuroblastoma

Tested agent/factor/ cellular state	Model	Cell line/experimental model	Outcome/ferroptotic mechanism affected	Reference
Buthionine sulfoximine (BSO)	*In vitro*	Post-AHSCT (CHLA-51, CHLA-79, CHLA-90, CHLA-134, and CHLA-136) ve pre-AHSCT (SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-BE(1), SK-N-DZ, SMS-LHN, LA-N-5, LA-N-6, SK-N-RA, SK-N-FI, LA-N-1, SK-N-SH, SK-N-AS and SMS-MSN) cell lines	Inhibition of GSH synthesis	(31)
Overexpression of mitochondrial ferritin	*In vitro*	SHSY-5Y cell line	Increased resistance against oxidative stress	(32)
Overexpression of mitochondrial ferritin	*In vitro*	Transgenic *drosophila* NB model	Suppression of ferroptosis	(33)
Silencing of Fe export protein (FPN)	*In vitro*	SH-SY5Y cell line	Accelerated ferroptosis by increasing iron-dependent lipid ROS accumulation	(34)
HDAC inhibitors	*In vitro*	SH-SY5Y cell line	Xc^- cystine transport inhibition (Ferroptosis induction effect in neuroblastoma cells while ferroptosis inhibition effect in neuronal cells)	(35)
Isoflurane	*In vitro*	SHSY-5Y cell line	Beclin1-SLC7A11 complex formation and inhibition cystine-glutamate antiporter	(36)
Withaferin-A	*In vitro*	IMR-32, SK-N-SH, Kelly, NB69, and CHP-134, NLF, SH-EP, SH-SY5Y, SK-N-AS, SK-N-BE(2) C, and SK-N-DZ NB cell lines	Induction of canonical (GPX4) and non-canonical (KEAP1, HMOX) ferroptosis	(37)
Withaferin-A and withaferin-A NP	*In vivo*	BALB/c nude mice NB xenograft model	Decreased GPX4 activity and induction of ferroptosis Inhibition of tumor growth	(38)
Sulphasalazine and auranofin	*In vivo*	Patient-derived xenograft (PDX) NB model	Induction of ferroptosis by targeting the Xc-cystine/GSH pathway	(39)
Sulphasalazine	*In vivo*	HTLA-230/HTLA-ER NB CSC	Decrease of intracellular GSH levels, Switch from oxidative phosphorylation to aerobic glycolysis, Downregulation of GPX4 activity, Induction of ferroptosis by lipid peroxidation	(40)
C2-4 (PKCα inhibitor)	*In vitro*	HTLA-230/HTLA-ER CSC	Decrease of intracellular GSH levels, Switch from oxidative phosphorylation to aerobic glycolysis, Downregulation of GPX4 activity, Induction of ferroptosis by lipid peroxidation	(41)
Sulphasalazine	*In vitro*	LAN5, KELLY, LAN1, SKNSH, CHP134, CHP212, IMR32, SKNAS, SKNBE, SKNFI, SMSKAN, SMSKANR, SMSKCN, SMSKCNR, MYCN2, SHEP21N NB cell lines	Sensitivity to ferroptosis by ROS formation	(41)

AHSCT: Autologous hematopoietic stem cell transplantation, BSO: Buthionine sulphoximine, CSC: Cancer stem cells, FPN: Ferroportin, GPX4: Glutathione peroxidase 4, GSH: Glutathione, HDAC: Histone deacetylase, HMOX: Heme oxygenase, KEAP1: Kelch-like ECH-related protein 1, NB: Neuroblastoma, NP: Nanoparticle, ROS: Reactive oxygen species, SAS: Sulphasalazine, SLC7A11: Solute carrier family 7 member 11

amplified NB can be evaluated as a potential treatment approach.

Ethics

Authorship Contributions

Concept: G.S., Z.A., N.O., Design: G.S., Z.A., N.O., Data Collection or Processing: G.S., Literature Search: G.S., Writing: G.S., Z.A.

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