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Time-dependent exposure to venetoclax induces ferroptosis in human neuroblastoma cells via upregulated transferrin gene expression and lipid peroxidation

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MAIN POINTS

- This study demonstrated for the first time that venetoclax induces ferroptosis in human neuroblastoma cells.
- Venetoclax alters intracellular iron homeostasis by upregulating the expression of transferrin and downregulating the expression of ferroportin1, resulting in enhanced labile iron pool content.
- Venetoclax increases oxidative stress and lipid peroxidation in neuroblastoma cells, which are inhibited by the iron chelator deferoxamine.

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ABSTRACT

Aim: Neuroblastoma is one of the most widely diagnosed extracranial tumors in pediatric patients with poor survival rates. Despite the available treatment options, alternative treatment strategies are required, especially for high-risk patients. Venetoclax (VTX) is a small-molecule inhibitor of the anti-apoptotic protein Bcl-2, originally approved for the treatment of acute myeloid and chronic lymphocytic leukemia. Mounting evidence indicates that VTX may also be a promising agent against other types of cancer. However, data on the alternative mechanisms of VTX toxicity are limited. The present study aimed to unveil the potential of the agent against neuroblastoma. The effect of VTX on iron metabolism and ferroptotic cell death in neuroblastoma cells was investigated for the first time.

Materials and Methods: Cell viability was determined using the MTT assay. Oxidative stress, intracellular iron content, and lysosomal integrity were visualized using confocal microscopy. The MDA assay was performed to detect lipid peroxidation. The expression of TFR, FPN1, and GPX4 was determined by RT-qPCR.

Results: VTX significantly reduced cell viability in a time- and concentration-dependent manner, which was reversed by the addition of ferroptosis inhibitors. Further experiments revealed that VTX induces ROS generation, leads to lysosomal degradation and iron accumulation followed by lipid peroxidation, all of which are ferroptosis markers. RT-qPCR analyses indicated that VTX upregulates TFR gene expression while downregulating GPX4 and FPN1.

Conclusion: The present study demonstrated for the first time that VTX activates ferroptotic pathways in neuroblastoma cells and may be considered as a promising agent for add-on therapies.

Keywords: Ferroptosis, Iron, Neuroblastoma, Oxidative stress, Venetoclax

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INTRODUCTION

Neuroblastoma is a common pediatric malignant tumor that originates from neuronal progenitor cells [1]. Current treatment options include surgery, chemotherapy, and radiotherapy. However, high-risk patients have a poor prognosis with an overall survival rate of 50%. Therefore, it is crucial to develop novel treatment options with higher efficacy [2].

Venetoclax (VTX) is a small molecule that was first developed as a selective Bcl-2 inhibitor against acute myeloid leukemia (AML). FDA approval for relapsed or refractory chronic lymphocytic leukemia (CLL) was obtained in 2016 [3]. Bcl-2 is often highly expressed in cancer cells, including lung and hematological cancers [4,5]. Similarly, neuroblastoma cells usually manage to escape apoptosis via increased Bcl-2 expression, indicating that these tumors may be more sensitive to Bcl-2 inhibitors [6]. Thus, navitoclax was first developed as a potent Bcl-2 inhibitor. However, it was less selective to Bcl-2 and caused severe thrombocytopenia [7]. Therefore, VTX was developed as a more selective Bcl-2 inhibitor that did not cause thrombocytopenia and was well tolerated by patients. Even though VTX was found to be weak as a single agent, there are reports demonstrating increased sensitiv-

ity to chemotherapy following the addition of VTX in conventional regimens [8,9]. On the other hand, preclinical studies have also shown that the upregulation of Bcl-2 may cause resistance to the agent [10]. Discovering alternative mechanisms of VTX may provide more effective treatment approaches in cancer.

Ferroptosis is a distinct type of regulated cell death mechanism discovered in 2012 by Dixon et al. in 2012 [11]. Unlike apoptosis and autophagy, intracellular iron accumulation, oxidative stress, and iron-dependent lipid peroxidation are considered hallmarks of this unique cell death [12]. The different mechanisms of ferroptosis make it particularly advantageous against cancer cells resistant to other cell death pathways. Numerous studies have reported that ferroptosis activation significantly increases cancer cell susceptibility to chemotherapy [13]. Hence, developing new approaches to treatment has become a trending focus of cancer research. Yu et al. [14] reported that VTX may have the potential to induce ferroptotic pathways in AML [14]. However, its effect on iron metabolism or ferroptotic pathways in neuroblastoma cells has not been investigated.

The present study aimed to shed light on the alternative pathways of VTX. The ferroptotic activity of VTX in human neuroblastoma cells was determined for the first time. The status of iron and lipid-related markers was investigated following VTX treatment, and the observed cytotoxic effect of the agent was compared with that of L929 healthy cells. Due to the multiple advantages such as tolerability, low risk of thrombocytopenia and ability to cross the blood-brain barrier, VTX may be a potential candidate for add-on therapies in neuroblastoma [15].

MATERIALS AND METHODS

Cell culture, Chemicals, and Experimental design

SH-SY5Y neuroblastoma and L929 fibroblast cells were purchased from the American Type Culture Collection (ATCC) (USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Sigma-Aldrich (Germany). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin were obtained from Capricorn Scientific (Germany). Venetoclax was obtained from BLD Pharm (China). Trolox, mannitol, N-acetylcysteine, ferrostatin-1, and deferoxamine were purchased from Sigma-Aldrich (Germany). PCR primers were obtained from Sentromer DNA Technologies (Turkey).

SH-SY5Y and L929 cells were maintained in high-glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine. Both cell lines were incubated in a humidified atmosphere with 5% CO_2 at 37°C. Venetoclax was dissolved in dimethyl sulfoxide (DMSO) and diluted in DMEM to prepare various concentrations. The final DMSO concentration did not exceed 0.1%. Untreated cells were used as controls. Each experiment was performed in triplicate, and cell viability, oxidative stress, and ferroptotic hallmarks were evaluated.

In vitro cell viability

The effect of VTX on cell viability was evaluated using the MTT assay. Cells were seeded in 96-well plates and incubated overnight. Then, VTX (0.5-50 μM) was administered in the presence or absence of scavengers (trolox, mannitol, and N-acetylcysteine) and ferroptosis inhibitors (ferrostatin-1 and deferoxamine). After 24 h of incubation, the MTT reagent (5 mg/ml) was added to fresh medium for 3 h. The resulting formazan particles were dissolved in SDS-HCl, and the absorbance was measured at 540 nm using a microplate reader (Thermo, Germany).

ROS Generation

The state of intracellular ROS following VTX treatment was determined using a 2',7'-dichlorofluorescein diacetate fluorescent (DCFH-DA) probe. Cells were exposed to VTX in the presence or absence of deferoxamine, mannitol, ferrostatin-1, or N-acetylcysteine. Then, the plate was washed twice with 1X PBS and incubated for 30 min with DCFH-DA (20 μM) and Hoechst 33342 (1 μg/ml) probe prepared in serum-free medium. After a final wash of 3 times with 1X PBS, the acquired green fluorescence was visualized with a confocal microscope at wavelengths of 361⁄497 nm (ex/em) and 488/535 nm (ex/em) (LSM 900, Carl Zeiss, Germany). Each experiment was performed in at least 2 replicates, taking 4 images from each group (10X) [16].

Determination of lipid peroxidation

Lipid peroxidation in VTX-treated neuroblastoma cells was determined by measuring the end-product, malondialdehyde (MDA). Before the experiment, cells were seeded in culture plates and incubated overnight. Following the administration of VTX (25 μM) for 24 h, the cells were harvested and lysed in cell lysis buffer (Cell Signaling, Germany). The lysates were used for the assay according to the TBARS method [17]. The absorbance of the acquired pink color was measured at 532 nm using a microplate reader (MultiskanSky, Thermo, Germany).

Detection of labile iron pool and lipid accumulation

Intracellular iron content and lysosomal integrity were visualized by calcein-acetoxymethyl ester (calcein-AM) and neutral red (NRU) staining [18]. VTX-treated cells were washed twice with 1X PBS and then incubated for 30 min with calcein-AM (10 μM), neutral red (10 μM), and Hoechst 33342 (1 μg/ml) probe in serum-free medium. After the final wash, images of the acquired fluorescence were captured using confocal microscopy at 495⁄515 nm (ex/em), 470/580 nm (ex/em), and 488/535 nm (ex/em) (LSM 900, Carl Zeiss, Germany). Experiments were conducted in at least 2 replicates, taking 4 images from each group (10X).

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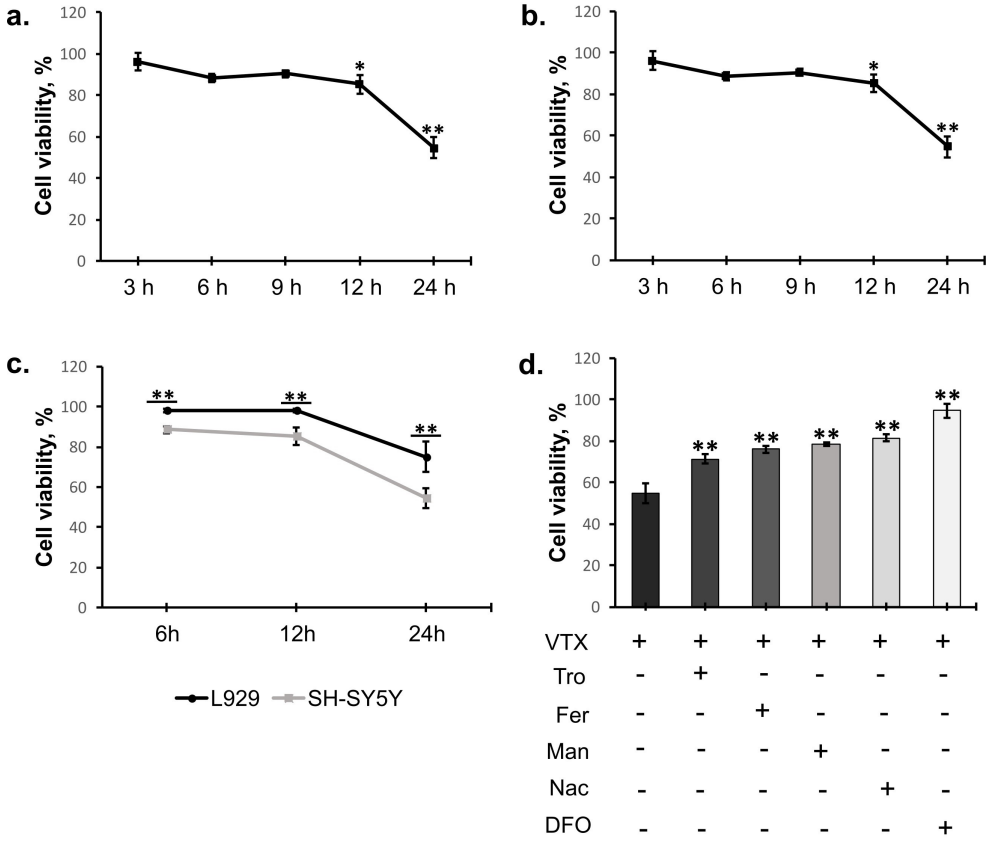

Figure 1. The cytotoxic effect of VTX on human neuroblastoma cells. (a) SH-SY5Y cells were treated with VTX (0.5-50 µM) for 24 h and the cell viability was determined with MTT assay. (b) The time-dependent effect of VTX (25 µM) on neuroblastoma cell viability. (c) The comparison of VTX toxicity between SH-SY5Y neuroblastoma cells and L929 normal cells. (d) Cells were treated with VTX (25 µM) alone and in the presence of scavengers (trolox, mannitol, n-acetylcysteine) and ferroptosis inhibitors (ferrostatin-1, deferoxamine), and the viable cells were detected with MTT assay. Data represent the average of at least 4 replicates, *p<0.01, **p<0.001. VTX: Venetoclax, Tro: Trolox, Fer: Ferrostatin-1, Man: Mannitol, Nac: N-acetylcysteine, DFO: Deferoxamine.

RT-qPCR

RNA was extracted using TRIzol reagent (ABP Biosciences, USA) according to the manufacturer's instructions. cDNA from each sample was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). RT-qPCR was performed using SYBR Green (Wizbiosolutions Inc., Korea) according to the manufacturer's protocol. The PCR conditions were as follows: 1 cycle of 95 oC 5 mins, 40 cycles of 95 oC 30 seconds and 65 oC 60 seconds, 1 cycle of 95 oC 5 seconds. Data analysis was performed using ΔΔCt method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primer sequences for each gene are provided in Supplementary Table 1.

Statistical analysis

Data obtained from the experiments were analyzed using SPSS 20.0 (IBM Co, USA, 5725-A54). One-way analysis of variance (ANOVA) was employed for multiple comparisons, followed by Tukey's test. The difference between the two groups was determined using Student's t-test. All data are expressed as mean ± standard deviation (SD) with $p<0.05$ being considered statistically significant.

RESULTS

Venetoclax exhibits a significant cytotoxic effect on neuroblastoma cells in a time- and concentration-dependent manner

SH-SY5Y neuroblastoma cells were treated with 0.5, 1, 5, 10, 25 and 50 µM VTX and the viable cells were determined after 24 h Results showed that VTX was significantly toxic at 25 and 50 µM with 54.70 ± 4.94 and 34.06 ± 8.82 (%) viability ($p=0.000013$ for both values) (Figure 1a). Time-dependent activity of the minimum toxic concentration of the agent (25 µM) revealed that the cytotoxic effect starts at 12 h and increases over 24 h (Figure 1b). Compared with L929 healthy cells, a significant selectivity was observed in all time periods (Figure 1c). To understand whether VTX-induced cell death is related to ferroptosis, several inhibitors were applied along with the agent. The results indicated that the antioxidants trolox (25 µM), mannitol (10 mM), N-acetylcysteine (1 mM), ferroptosis inhibitors ferrostatin-1 (1 µM), and deferoxamine (250 µM) reversed the effect of VTX, with the ferrop-

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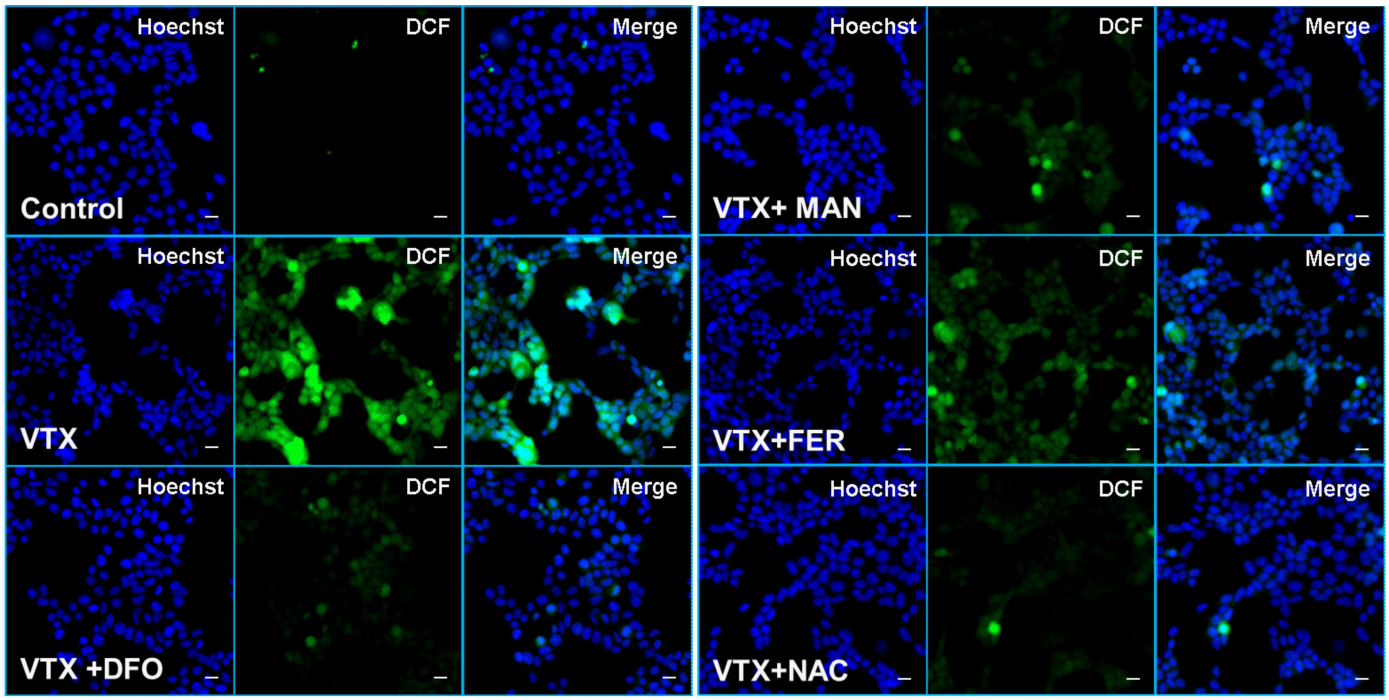

Figure 2. Visualization of ROS generation in SH-SY5Y cells treated with VTX (25 μM) alone or in combination with inhibitors for 12 h Cells were stained with DCF, and fluorescence emission was detected using confocal microscopy. VTX: Venetoclax, DFO: Deferoxamine, MAN: Mannitol, FER: Ferrostatin-1, NAC: N-acetylcysteine.

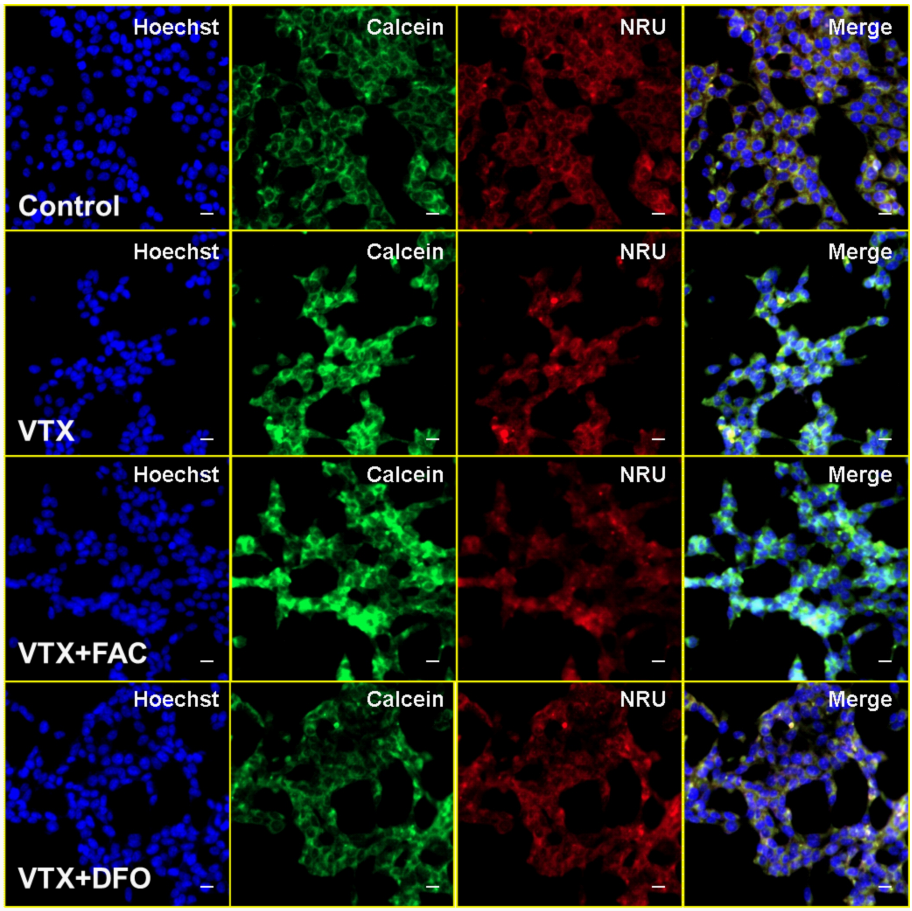

Figure 3. Imaging the enhanced iron and lysosomal degradation in SH-SY5Y neuroblastoma cells treated with VTX (25 μM) in the presence of ferric ammonium citrate (FAC) or iron chelator deferoxamine (DFO) for 12 h. Iron was detected with calcein staining, and lysosomal degradation was visualized with neutral red staining using confocal microscopy.

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tosis inhibitor deferoxamine achieving the best reversal (p = 0.000024) (Figure 1d).

ROS generation, iron and lysosomal degradation increased following venetoclax treatment

The state of oxidative stress in VTX-treated cells was visualized using confocal microscopy. The cells were treated with VTX (25 μM) for 12 h and stained with DCF. The acquired images indicated an apparent increase in ROS generation in the VTX-treated group compared with the untreated control, which was clearly inhibited by the addition of ferroptosis inhibitors and scavengers (Figure 2). Next, the iron content and lysosomal integrity of the cells were detected with Hoechst/calcein/neutral red triple staining, where calcein showed a labile iron pool and neutral red showed lysosomal integrity, simultaneously. The results indicated that both iron and lysosomal degradation increased following VTX treatment compared to the untreated control group. Similar results were observed with the addition of FAC, which was suppressed by deferoxamine (Figure 3).

Venetoclax induces iron-dependent lipid peroxidation

To investigate the lipid peroxidation status in cells treated with VTX, the end-product MDA levels were measured in different time periods using the TBARS method. Following 12 and 24 h of incubation with VTX (25 μM), MDA levels were significantly higher than those in the control group, by 134.02 ± 2.00 and 204.83 ± 7.84 (%), respectively (p = 0.006264) (Figure 4a). Further experiments revealed that scavengers and ferroptosis inhibitors suppressed the increased lipid peroxidation, with the highest suppressive effect observed by deferoxamine (p = 0.005496) (Figure 4b).

TFR, FPN1, and GPX4 gene expressions were significantly altered in Venetoclax-treated neuroblastoma cells

Cells were administered with VTX for 12 h, and the mRNA expression of iron-related genes, TFR and FPN1, and the cellular antioxidant enzyme GPX4 were analyzed by RT-qPCR. The results revealed a significant upregulation of TFR, which was detected as approximately 2-fold, whereas FPN1 was downregulated by 5-fold and GPX4 was downregulated by 1.5-fold ($p<0.05$).

DISCUSSION

Neuroblastoma is an extracranial pediatric malignant tumor observed in approximately 1 in 10 children. Due to the low survival rates in high-risk patients, extensive research has been conducted on alternative therapeutics [19]. Venetoclax is primarily used against hematological cancers and has recently drawn attention for its potential in different types of cancers. Several studies have reported that it exhibits antitumoral behavior in glioma and lung and breast cancers [20–22]. Furthermore, a case study reported that VTX may contribute to neuroblastoma treatment when combined with conventional chemotherapeutics [8]. However, VTX is mostly known for its Bcl-2 inhibitory effect, and data on its alternative mechanisms of action are very limited. Deciphering these pathways induced by VTX, beyond Bcl-2 inhibition, may provide a more effective and rational combinatorial therapy regimen.

The ferroptotic activity of VTX was investigated in neuroblastoma cells. First, the concentration-dependent effect of the agent was determined and 25 μM was selected for further experiments because it yielded an approximate IC_{50} value (Figure 1a). Time-dependent cell viability analysis demonstrated that 25 μM of VTX causes a significant death starting from 12 h, which gradually increases over 24 h (Figure 1b). Furthermore, it was observed that some antioxidants reversed the cytotoxic effect of VTX, which correlates with the ROS-generating role of the agent [14]. However, the iron chelator and ferroptosis inhibitor deferoxamine exhibited the highest inhibition rate, indicating that iron is a major contributor to VTX-induced cell death (Figure 1d). To confirm this, confocal microscopy was used to visualize the labile iron pool in VTX-treated cells, which reflected an obvious increase in the iron content. Similar results were obtained when the cells were treated with FAC, which is an iron source. To investigate the role of iron in VTX-induced cell death, neuroblastoma cells were exposed to both VTX and FAC, and neutral red staining was performed to highlight the integrity of lysosomes. As shown in Figure 3, both VTX and FAC aggravated lysosomal degradation. The close relationship between lysosomal function and ferroptosis is well established. Because lysosomes contain iron and proteolytic enzymes, the breakdown of lysosomal membranes results in the release of iron, which contributes to the increase in the labile iron pool [23].

Oxidative stress and iron-dependent lipid peroxidation are among the essential hallmarks of ferroptotic cell death, which may be induced by VTX [14,20]. In this study, ROS generation was detected using DCF staining. The acquired images showed that VTX treatment enhances the available ROS in cells. Generally, cancer cells increase their antioxidant defense capacity to overcome cellular damage caused by excess ROS. Glutathione peroxidases (GPx) are major players in this regard because they neutralize free radicals [24]. GPx4 is a critical suppressor of ferroptotic cell death; thus, downregulation of this enzyme is particularly important to enable ROS generation and the subsequent events [25]. RT-qPCR analyses demonstrated that 12 h of VTX treatment significantly suppressed GPx4 gene expression in neuroblastoma cells, which may explain the increase in ROS generation. Excess ROS in the cellular environment damage lipid–based structures, especially polyunsaturated fatty acids (PUFAs) found in cell membranes, leading to lipid peroxidation [26]. To verify this, the end-product of lipid peroxides (MDA) was measured following VTX treatment. The results demonstrated a significant elevation in MDA levels in a time-dependent manner, which correlated with the results of the previous experiments (Figure 4a). To confirm that lipid peroxides were iron-dependent,

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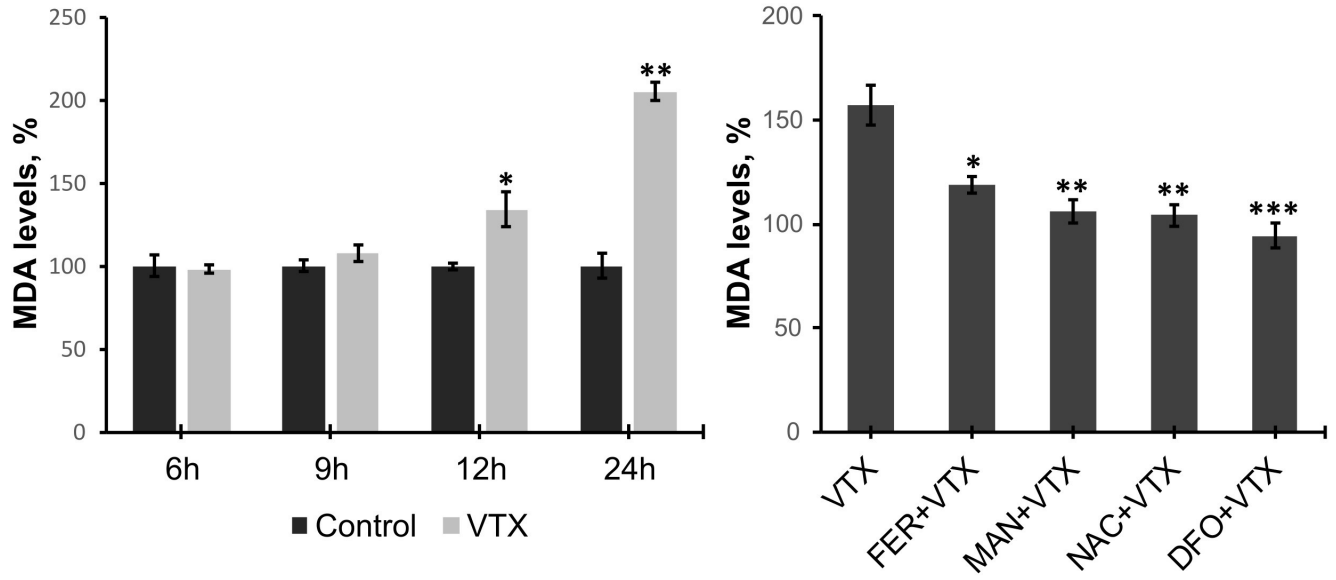

Figure 4. Detection of lipid peroxidation in SH-SY5Y cells. (a) Following VTX (25 μM) treatment, cells were lysed, and the end-product of lipid peroxidation, malondialdehyde (MDA), was measured using the TBARS method. (b) The effect of VTX (25 μM) combined with scavengers and ferroptosis inhibitors on lipid peroxidation. Data represent the average of at least 3 replicates, *p<0.01, **p<0.001, ***p<0.0001. VTX: Venetoclax, FER: Ferrostatin-1, MAN: Mannitol, NAC: N-acetylcysteine, DFO: Deferoxamine.

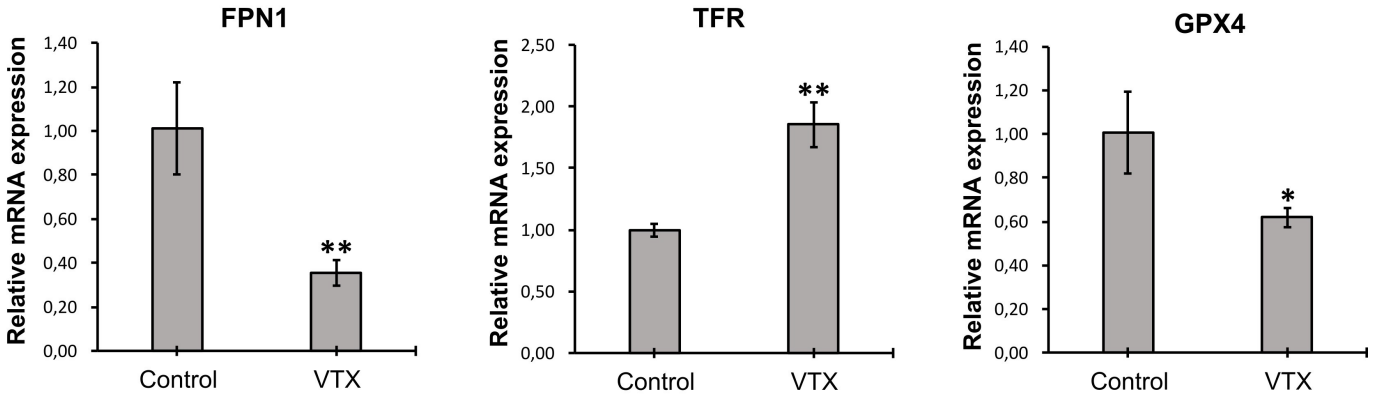

Figure 5. Gene expression analysis using RT-qPCR Cells were treated with VTX (25 μM), and FPN, TFR, and GPX4 mRNA expression was detected after 12 h Data represent the average of at least 3 replicates, *p<0.01, **p<0.001. FPN1: Ferroportin, TFR: Transferrin, GPX4: Glutathione peroxidase-4.

VTX was administered to neuroblastoma cells in the presence of deferoxamine and antioxidants. The findings indicated that deferoxamine suppresses the formation of MDA more than the antioxidants, leading to the conclusion that iron mediates VTX-induced lipid peroxidation (Figure 4b).

Iron is a critical player in ferroptotic cell death [27]. Hence, iron metabolism and associated elements in the cellular environment must be explored to confirm the activation of this pathway. In this regard, transferrin (TFR) and ferroportin1 (FPN1) are among the major regulators of cellular iron metabolism. TFR allows iron entry into the cell, while FPN1 mediates iron export. In ferroptosis, TFR expression is upregulated and FPN1 is downregulated to enrich the intracellular iron content [28]. TFR and FPN1 gene expressions were analyzed in VTX-exposed neuroblastoma cells. After 12 h of treatment, TFR gene expression increased by approximately 2-fold, whereas FPN1 expression was downregulated by 5-fold (Figure 5). These findings support the hypothesis that VTX activates ferroptotic pathways in neuroblastoma cells.

Despite the promising data obtained in this study, some limitations should be addressed in future studies. The potential of VTX as an add-on therapy, such as whether VTX would exhibit ferroptotic activity when combined with chemotherapeutics, should be explored.

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Furthermore, the long-term effects of VTX use, especially on healthy tissues, should be investigated.

Conclusion

Neuroblastoma is a common neoplasm in children with insufficient treatment options. VTX is a selective Bcl-2 inhibitor that may be a promising treatment candidate. In this study, the ferroptotic behavior of VTX was investigated in neuroblastoma cells for the first time. The data obtained indicated that VTX exposure increased oxidative stress and iron-dependent lipid peroxidation, which are hallmarks of ferroptosis. In addition, iron metabolism was altered with upregulated transferrin and downregulated ferroportin gene expression following VTX administration. Taken together, the findings of this study shed light on the alternative mechanisms of VTX, demonstrating its potential contribution to the treatment of neuroblastoma.

Ethics Committee Approval: It is a cell study that does not require ethics committee approval.

Informed Consent: Not necessary for this manuscript.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that they have no known competing interests or personal relationships that could have influenced the work reported in this paper.

Author Contributions: Concept: ZE, EO; Design: ZE, EO; Supervision: ZE, EO; Materials: ZE; Data Collection and/or Processing: ZE, EO; Literature Review: EO; Writing: ZE, EO; Critical Review: ZE, EO.

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