Deferasirox combination with eltrombopag shows anti-myelodysplastic syndrome effects by enhancing iron deprivation–related apoptosis

Lei Huang , Mengyue Tian, Zhaoyun Liu , Chunyan Liu, Rong Fu

Hematology, Tianjin Medical University General Hospital, Tianjin, China

Correspondence to
Dr Rong Fu, Hematology, Tianjin Medical University General Hospital, Tianjin 300052, China; furong8369@tmu.edu.cn

LH and MT contributed equally.

Accepted 9 November 2021
Published Online First 17 December 2021

ABSTRACT

Iron overload (IO) affected the survival of patients with myelodysplastic syndrome (MDS). Deferasirox (DFX) is widely used in patients with MDS for iron chelation therapy, but is not suitable for MDS patients with severe thrombocytopenia. Eltrombopag (ELT) is a type of thrombopoietin receptor (TPOR) analog used in the treatment of thrombocytopenia. Therefore, we sought to explore the synergistic effects and possible mechanisms of DFX combination with ELT in MDS cells. In our study, the combination of DFX with ELT synergistically inhibited proliferation, induced apoptosis and arrested cell cycle of MDS cells. Through the RNA-sequence and gene set enrichment analysis (GSEA), iron metabolism–related pathway played important roles in apoptosis of SKM-1 cells treated with DFX plus ELT. Transferrin receptor (TFRC) was significantly highly expressed in combination group than that in single agent groups, without affecting TPOR. Furthermore, the apoptosis of the combination group MDS cells could be partially reversed by ferric ammonium citrate (FAC), accompanied with decreased expression of TFRC. These results suggested that the combination of DFX and ELT synergistically induced apoptosis of MDS cells by enhancing iron deprivation–related pathway.

Significance of this study

What is already known about this subject?

- Anemia is common in patients with myelodysplastic syndrome (MDS), and repeated blood transfusions and ineffective hematopoiesis may cause iron overload (IO).
- Iron chelation therapy plays important roles in delaying the progression of patients with MDS.
- Deferasirox (DFX) is an oral iron chelator, but is of limited used in MDS patients with thrombocytopenia, while eltrombopag (ELT) could improve thrombocytopenia.
- ELT could significantly reduce the ferritin level of aplastic patients through iron chelation effects.

What are the new findings?

- The combination of DFX and ELT shows synergistic anti-MDS effects at suitable drug concentrations.
- DFX combined with ELT inhibits proliferation, arrests cell cycle and induces apoptosis of MDS cells.
- Enhancing iron deprivation–related apoptosis might be the mechanism of the combination in vitro experiments.

How might these results change the focus of research or clinical practice?

- DFX combined with ELT plays synergistic roles in anti-MDS cells by enhancing iron deprivation–related apoptosis, providing a new way for treatment of IO MDS patients with thrombocytopenia.

INTRODUCTION

Among patients with myelodysplastic syndrome (MDS), about 60%–80% have anemia at the early stage of disease, and nearly 90% need blood transfusion during the course of the disease progression.[1] Iron overload (IO) is caused by ineffective hematopoiesis and repeated blood transfusions in the majority of patients with MDS, and affect bone marrow hematopoiesis through various mechanisms.[2] Multiple studies have shown that MDS patients with IO had poor prognosis, while iron chelation therapy could significantly improve bone marrow hematopoiesis, prolong the overall survival (OS) of patients with MDS, and reduce the risk of transformation to acute myeloid leukemia (AML).[3 4] Deferasirox (DFX) has been widely used in patients with IO MDS for iron chelation therapy in recent years, which could significantly increase the OS of patients with MDS.[5] DFX improves the bone marrow hematopoiesis of patients with MDS through a variety of mechanisms and reduces the risk of MDS transformation to AML.[4 6 7] DFX is not generally used in patients with high-risk MDS, platelet counts below 50×10^9/L, or poor performance.[8] Therefore, a reasonable combination therapy is needed for IO MDS patients with thrombocytopenia.

Eltrombopag (ELT) is a type of small-molecule thrombopoietin receptor (TPOR) agonist, which acts on the transmembrane structure region of TPOR receptor, induces proliferation and differentiation of megakaryotic progenitor

To cite: Huang L, Tian M, Liu Z, *et al*. *J Investig Med* 2022;**70**:953–962.

cells, and promotes platelet generation.[9] Previous studies have shown that ELT did not promote malignant clonal proliferation in patients with AML and MDS.[10] In contrast, ELT combined with demethylated drugs could improve platelet counts in patients with MDS and play potential anti-leukemia effects without increasing the side effects of drugs and the risk of leukemia transformation.[11 12] Due to the limited use of DFX in IO MDS patients with thrombocytopenia, whether ELT combined with DFX could play synergistic effects in anti MDS cells, and improve thrombocytopenia in MDS patients is still unknown.

In our study, we try to determine whether the combination of DFX and ELT could synergistically induce apoptosis of MDS cells and the exact molecular mechanisms involved in the combination group, in order to provide evidence for the use of DFX combination with ELT in patients with high-risk MDS.

MATERIALS AND METHODS

Cell culture and reagents

Human MDS cell lines SKM-1 and MUTZ-1 were cultured in 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 mg/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco) at 37°C in a 5% CO_2 atmosphere. SKM-1, a cell line established from the patient progression to myelomonocytic leukemia with p53 chromosomal abnormalities, was obtained from the Health Science Research Resources Bank in Japan.[13] MUTZ-1, a cell line established from childhood MDS with del(5q), was obtained from the German Brauschweig cell center.[14] For treatments with DFX or ELT (TargetMol), fresh solutions were prepared before every experiment.

Cell proliferation assays

2×10^4 cells seeded in 96-well plates were treated with DFX and/or ELT for 48 and 72 hours, 10 µL CCK8 (Engreen, China) was added to each well and incubated for another 3 hours at 37°C in a 5% CO_2 atmosphere. The absorbance of the samples was measured against a background control at 450 nm. Cell inhibition rate (%)=(OD450 of treatment group−OD450 of blank)/(OD450 of control group−OD450 of blank)×100%.

Cell apoptosis analysis

4×10^5 cells seeded in 6-well plates were treated with DFX, ELT or combination for 48 and 72 hours. 10^5 cells were resuspended in 200 µL staining solution containing 5 µL Annexin V–FITC and 5 µL propidium iodide (PI) (FITC-Annexin V staining kit, BD) for 30 min in the dark. Cell apoptosis was detected by flow cytometry (Beckman CytoFLEX), and analyzed by the CytExpert V.2.3 software platform.

Cell cycle analysis

After treatments with DFX, ELT or combination for 72 hours, cells were fixed in 70% ethanol for at least 4 hours at 4°C, and stained with 20 µg/mL PI containing 10 µg/mL RNase A for 30 min at room temperature. Cell cycle analysis was performed by flow cytometry. DNA distributions were analyzed by Kaluza C flow cytometry analysis software for the proportions of cells in the phases of the cell cycle.

The protocol of the RNA-sequence

The Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) was used to do the total RNA sample QC: RNA concentration, RIN value, 28S/18S and the fragment length distribution. The experimental workflow: (1) mRNA enrichment; (2) RNA fragment and reverse transcription; (3) end repair, add A-tailing and adaptor ligation; (4) PCR amplification; (5) denature and cyclization; (6) sequencing on BGISEQ-500 platform.

Detection of the surface marker CD71 and TPOR by flow cytometry

After treatments with DFX, ELT or combination for 72 hours, cells were incubated with 5 µL CD71 (555537, BD, USA) and 5 µL TPOR (562199, BD, USA) for 15 min at room temperature, washed twice with phosphate buffered solution (PBS), then immediately analyzed by flow cytometry, and analyzed by the CytExpert V.2.3 software platform.

Real-time qPCR

Total RNA was extracted from treated cells using TRIzol reagent (Tiangen Biotech, China). The first-strand cDNA was synthesized from 1 µg of total RNA using FastQuant RT Kit (Tiangen Biotech). Subsequently, RT-qPCR was performed in triplicate on the IQ5 (BIO-RAD, America) with SYBR Green and primers (10 µM), and the amplified-specific single product was validated by melt curve. The following gene-specific primer pairs were used: TFRC: Forward 5′-TGTGGCGTATAGTAAGGCTGCAAC-3′, Reverse 5′-GGCAATCCTGATGACCGAGATGG-3′; FTL: Forward 5′-GCGATGATGTGGCT CTGGAAGG-3′, Reverse 5′-TGT GGAGGTTGGTCAGGTGGTC-3′; BCL2: Forward 5′-CGACTTCGCCGAGATGTCCAG-3′, Reverse 5′-CGGTTCAGGTACTCAGTCATC CAC-3′; Caspase-3: Forward 5′-TTGTAGAAGTCTAACTGGAA-3′, Reverse 5′-CCA TGTCATCATCAACAC-3′; β-actin: Forward 5′-TGGACATCCGCAAAGACCTGT-3′, Reverse 5′-CACAC-GGAG TACTTGCGCTCA-3′.

Western blot analysis

Total cell lysates were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) (100:1), and protein concentrations were detected by bicinchoninic acid (BCA) protein assay kit (Sangon Biotech, China). Proteins (30 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGEs) and transferred electronically to nitrocellulose (NC) filter membranes (EASYBIO, China). NC membranes were blocked with 5% bovine serum albumin (BSA) for 1 hour and then incubated overnight at 4°C with the following primary antibodies: anti-AKT (#4691S), anti-p-AKT (#9271S), anti-Cyclin D1 (#2978S), anti-BCL2 (#4223T), anti-caspase-3 (#9668S), anti-cleaved caspase-3 (#9668S), anti-β-actin (#4970S), anti-p53 (#2527) (Cell Signaling Technology, Beverly, Massachusetts, USA). After washing three times with Tris-Buffered Saline and Tween20 (TBST), each membrane was incubated with anti-mouse IgG (GenScript A00160) or anti-rabbit IgG (GenScript A00098) as a secondary antibody

for 1 hour at room temperature. The expressions of target proteins were detected by enhanced chemiluminescence kits (Millipore, Billerica, Massachusetts, USA).

Statistical analysis

Data were expressed as mean±SD. One-way analysis of variance was used for statistical analyses. The paired t-test was used for the data treated with or without FAC. $p<0.05$ was considered statistically significant. The Combination Index (CI) of DFX plus ELT was calculated using Calcusyn. CI <1, CI=1 and CI >1 indicated synergism, additive and antagonism effects, respectively. SPSS V.21 was used to analyze the data, which was plotted with GraphPad Prism V.5.

RESULTS

The synergistic antiproliferative effects on MDS cells treated with DFX plus ELT

To evaluate the antiproliferative effects of DFX or ELT on MDS cells, MDS cells were treated with various concentrations of DFX or ELT for 48 or 72 hours. Both DFX and ELT showed dose-dependent growth inhibitory effects in MDS cells. The IC50 values (95% CIs) of DFX plus ELT in SKM-1 were 74.62 (53.03 to 105.00) µM and 56.21 (48.08 to 65.72) µM at 48 hours, 39.39 (32.16 to 48.25) µM and 43.25 (31.99 to 58.47) µM at 72 hours, respectively, and those in MUTZ-1 were 128.60 (109.10 to 151.60) µM and 132.50 (83.29 to 210.90) µM at 48 hours, 78.15 (70.16 to 87.05) µM and 79.98 (58.89 to 108.60) µM at 72 hours, respectively. The effects of DFX combination with ELT were evaluated in MDS cells, the Combination Index (CI) determined by Calcusyn were lower than 1 at 48 or 72 hours (as shown in figures 1A,B and 2A,B).

DFX combined with ELT significantly increased apoptosis of MDS cells

Since the combination of DFX with ELT had synergistic antiproliferation effects on MDS cells, we further analyzed the apoptotic effects of DFX plus ELT on them. For SKM-1, the highest apoptosis rate occurred in 40 µmol/L DFX plus 40 µmol/L ELT group (control: 10.51%±2.21%, DFX: 18.65%±2.90%, ELT: 22.99%±3.74%, DFX and ELT: 46.42%±4.16% at 48 hours, control: 9.20%±1.84%, DFX: 21.04%±0.94%, ELT: 25.30%±3.73%, DFX +ELT: 66.82%±8.07% at 72 hours) (as shown in figure 1C,D,F). Meanwhile, for MUTZ-1, the highest apoptosis occurred in 80 µmol/L DFX plus 80 µmol/L ELT group (control: 7.45%±2.89%, DFX: 18.66%±4.14%, ELT: 23.72%±2.56%, DFX and ELT: 35.87%±5.06% at 48 hours, control: 7.95%±2.30%, DFX: 35.85%±9.22%, ELT: 32.31%±6.06%, DFX +ELT: 62.82%±5.93% at 72 hours) (as shown in figure 2C,D,F). We also detected the apoptosis-related signal pathway, we found that the gene expression of caspase-3 was significantly increased in combined group, with significantly decreased BCL2 ($p<0.05$, as shown in figure 3A,B). Meanwhile, the protein levels were verified by western blot (as shown in figure 3C,D), which indicated that DFX plus ELT showed the synergistic apoptotic effects on MDS cells.

Cell cycle arrested at G0/G1 phase after treatments with DFX plus ELT in MDS cells

In order to investigate the effects of DFX plus ELT on cell cycle, cells at G0/G1, S and G2/M stages were isolated and analyzed by flow cytometry. Cell cycle analysis demonstrated a significant increase in the percentage of cells at G1 phase after treatments with DFX plus ELT, compared with single agent for both MDS cell lines, with a concomitant significant decrease at S phase in SKM-1 (figures 1E,G and 2E,G; the detailed data are shown in table 1). Meanwhile, the protein level of P53 increased in combination group than that in single agent groups in both MDS cells, with decreased Cyclin D1 (figure 3C,D), which meant that the cell cycle mechanisms played important roles in the synergistic effects of DFX plus ELT against MDS cells.

RNA-sequence analysis and altered iron metabolism in MDS cells

RNA-sequence was performed on SKM-1 cells with different treatments of DFX, ELT or combination. A total of 17,825 genes were detected and repeated three times for each group, among them, the comparisons of genes with significant differences between groups are shown in figure 4Aa. We obtained the differentially expressed genes through the comparison between control, DFX, ELT or combination group, and selected the genes fulfilling with the q value <0.0001 among them. (q value is the corrected p value. The smaller the q value is, the more significant the difference of gene expression is.) We obtained 1341 common upregulated genes by intersections in the list of upregulated genes of each group, while 9843 common downregulated genes were obtained by intersections in the list of downregulated genes of each group (as shown in figure 4Ab–c). Combination with GSEA gene function analysis (as shown in figure 4Ad,e), we found that the iron metabolism–related signal pathway played important roles in the inhibition of SKM-1 cells in combination group. Subsequently, iron metabolism–related gene expression of TFRC in combination group was significantly higher than that in single agent or control groups in MDS cells, with significantly downregulated gene expression of FTL ($p<0.05$, as shown in figure 4B,C). The expression of iron metabolism–related cell surface marker CD71 significantly upregulated in combination group ($p<0.05$), without affecting TPOR (as shown in figure 4D–G).

Decreased iron metabolism–related apoptosis after being treated with FAC

Due to the increased levels of TFRC and apoptosis in MDS cells treated with DFX plus ELT, iron rescue assay was used to investigate the role of iron metabolism in inducing apoptosis in combination group. MDS cells were treated with 2 mmol/L FAC for 24 hours to construct the IO MDS cell model. IO MDS cells were washed with cell culture medium and then treated with DFX plus ELT for another 72 hours. We found that the apoptosis and gene expression of caspase-3 in combination group with FAC significantly decreased compared with those without FAC, with significantly increased gene expression of BCL2 (as shown in figure 5A–C). Meanwhile, the gene and protein levels of TFRC were significantly decreased in combination group

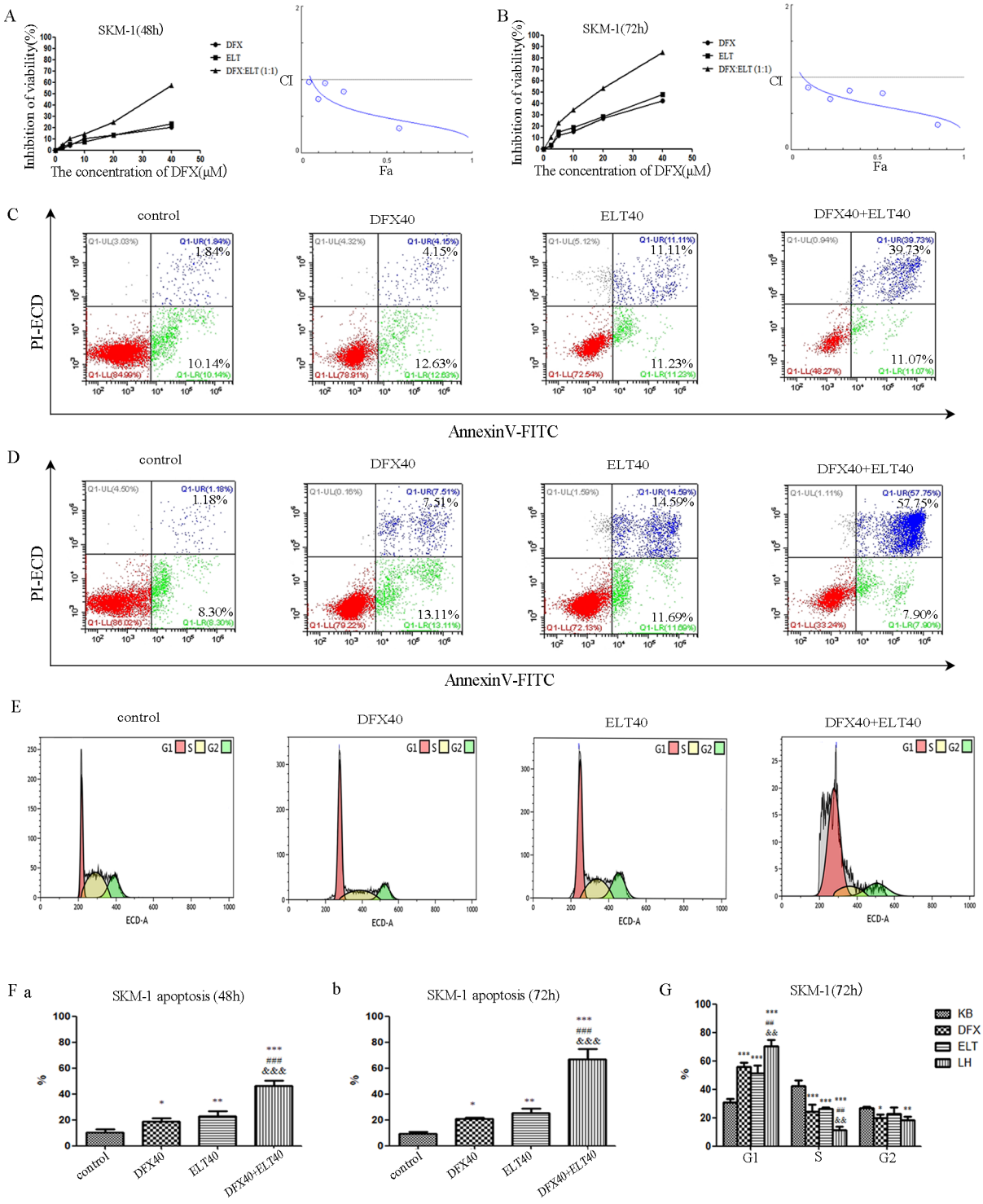

Figure 1 Deferasirox (DFX) and eltrombopag (ELT) synergistically inhibited cell proliferation, induced apoptosis and arrested cell cycle in SKM-1 at 48 and 72 hours. (A, B) The combination of DFX and ELT inhibited cell proliferation at 48 and 72 hours. Combination Index (CI) values for DFX plus ELT in SKM-1 were lower than 1. (C, D, F) The apoptosis of the combination in SKM-1 was significantly increased than that of control, DFX or ELT groups at 48 and 72 hours. (E, G) Cell cycle was arrested at G0/G1 phase after treatments with DFX plus ELT in SKM-1 at 72 hours. Compared with control group, * indicated p<0.05, ** indicated p<0.01, *** indicated p<0.001. Compared with DFX group, ## indicated p<0.01, ### indicated p<0.001. Compared with ELT group, && indicated p<0.01, &&& indicated p<0.001.

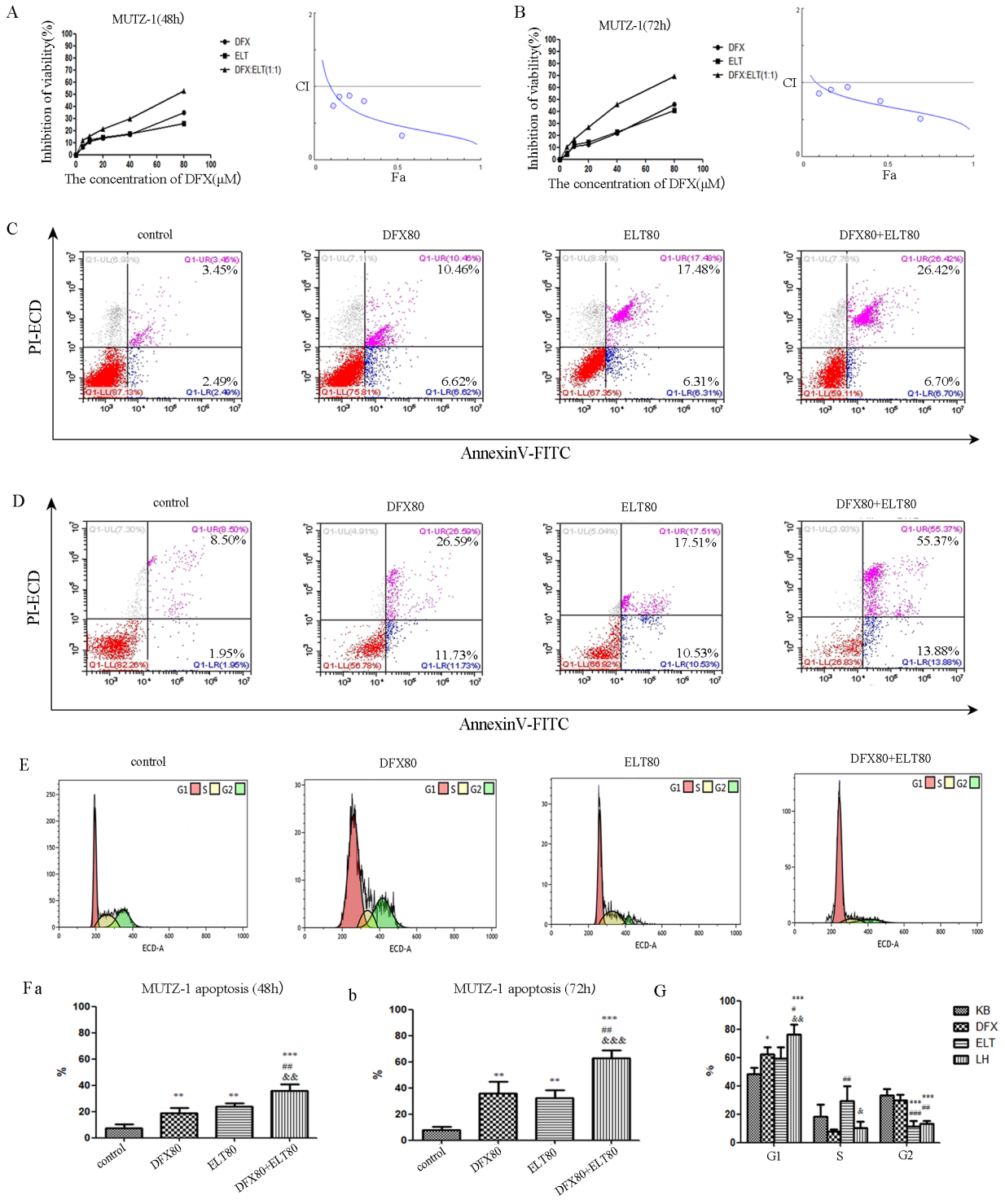

Figure 2 Deferasirox (DFX) and eltrombopag (ELT) synergistically inhibited cell proliferation, induced apoptosis and arrested cell cycle in MUTZ-1 at 48 and 72 hours. (A, B) The combination of DFX and ELT inhibited cell proliferation at 48 and 72 hours. Combination Index (CI) values for DFX plus ELT in MUTZ-1 were lower than 1. (C, D, F) The apoptosis of the combination in MUTZ-1 was significantly increased than that of control, DFX or ELT groups at 48 and 72 hours. (E, G) Cell cycle was arrested at G0/G1 phase after treatments with the combination in MUTZ-1 at 72 hours. Compared with the control group, * indicated p<0.05, ** indicated p<0.01, *** indicated p<0.001. Compared with the DFX group, ## indicated p<0.01, ### indicated p<0.001. Compared with the ELT group, & indicated p<0.05, && indicated p<0.01, &&& indicated p<0.001.

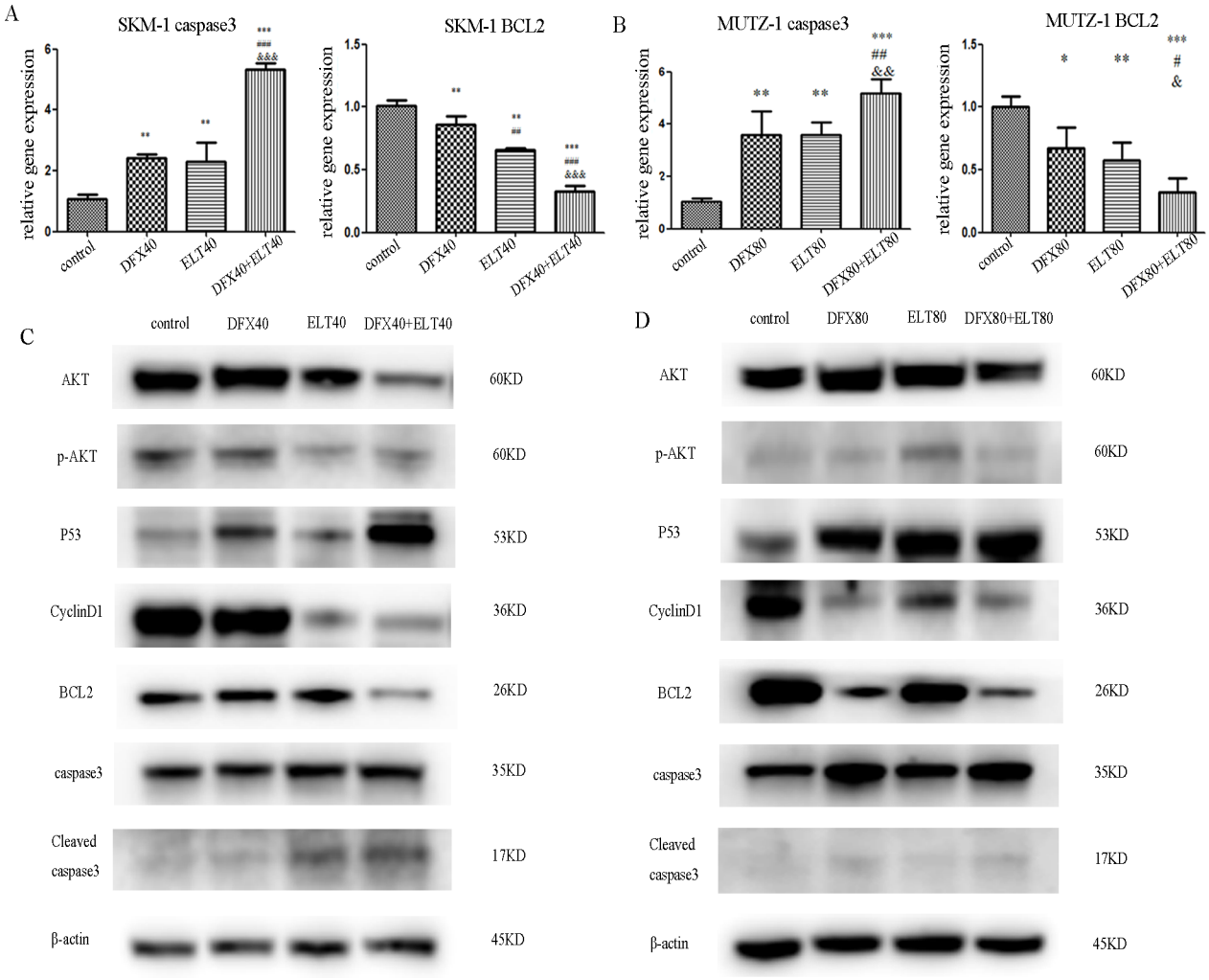

Figure 3 The signal pathways involved in deferasirox (DFX) plus eltrombopag (ELT) exerting anti-myelodysplastic syndrome (anti-MDS) effects at 72 hours. (A, B) The gene expression of caspase-3 significantly increased in combination group, with significantly decreased BCL2. (C, D) The combination group had the downregulation of BCL2 with upregulation of cleaved caspase-3. Meanwhile, the AKT in combination group was downregulated. The cell cycle–related protein P53 was upregulated in combination group, with downregulated Cyclin D1. Compared with the control group, * indicated p<0.05, ** indicated p<0.01, *** indicated p<0.001. Compared with the DFX group, # indicated p<0.05, ## indicated p<0.01, ### indicated p<0.001. Compared with the ELT group, & indicated p<0.05, && indicated p<0.01, &&& indicated p<0.001.

Table 1 The ratio of the cell cycle phase

	G1 phase (%)	S phase (%)	G2 phase (%)
SKM-1			
Control	30.81±2.62	42.40±3.69	26.79±1.17
DFX40	55.80±3.23	24.28±4.86	19.92±2.19
ELT40	51.20±5.43	26.09±1.01	22.71±4.42
DFX40 +ELT40	70.26±4.74	11.38±2.60	18.36±2.28
MUTZ-1			
Control	48.26±4.39	18.25±8.42	33.49±4.18
DFX80	62.28±4.87	7.96±1.20	29.76±3.85
ELT80	59.43±7.84	29.39±10.57	11.19±4.28
DFX80+ELT80	76.46±6.72	10.14±4.79	13.39±2.19

The data are presented as mean±SD.
DFX, deferasirox; ELT, eltrombopag.

with FAC in both MDS cells, with increased gene expression of FTL (p<0.05, as shown in figure 5D–F). The results above showed that the synergistic apoptosis effects of DFX plus ELT could be partially reversed by FAC.

DISCUSSION

IO affects the OS and prognosis of patients with MDS, while iron chelation therapy could reduce the cytotoxicity and DNA damage induced by excessive iron.[7 15] Although the ferritin significantly decreased in low or Int-1 risk IO MDS patients treated with DFX, but 79.8% of the patients terminated the treatment, 24.8% among them due to drug side effects or disease progression.[16] DFX is not generally used in patients with high-risk MDS, platelet counts of 50×10^9/L, or poor performance.[8] However, thrombocytopenia is common in patients with MDS and is usually associated with life-threatening bleeding complications. Recent studies have shown that 47% low or Int-1 MDS

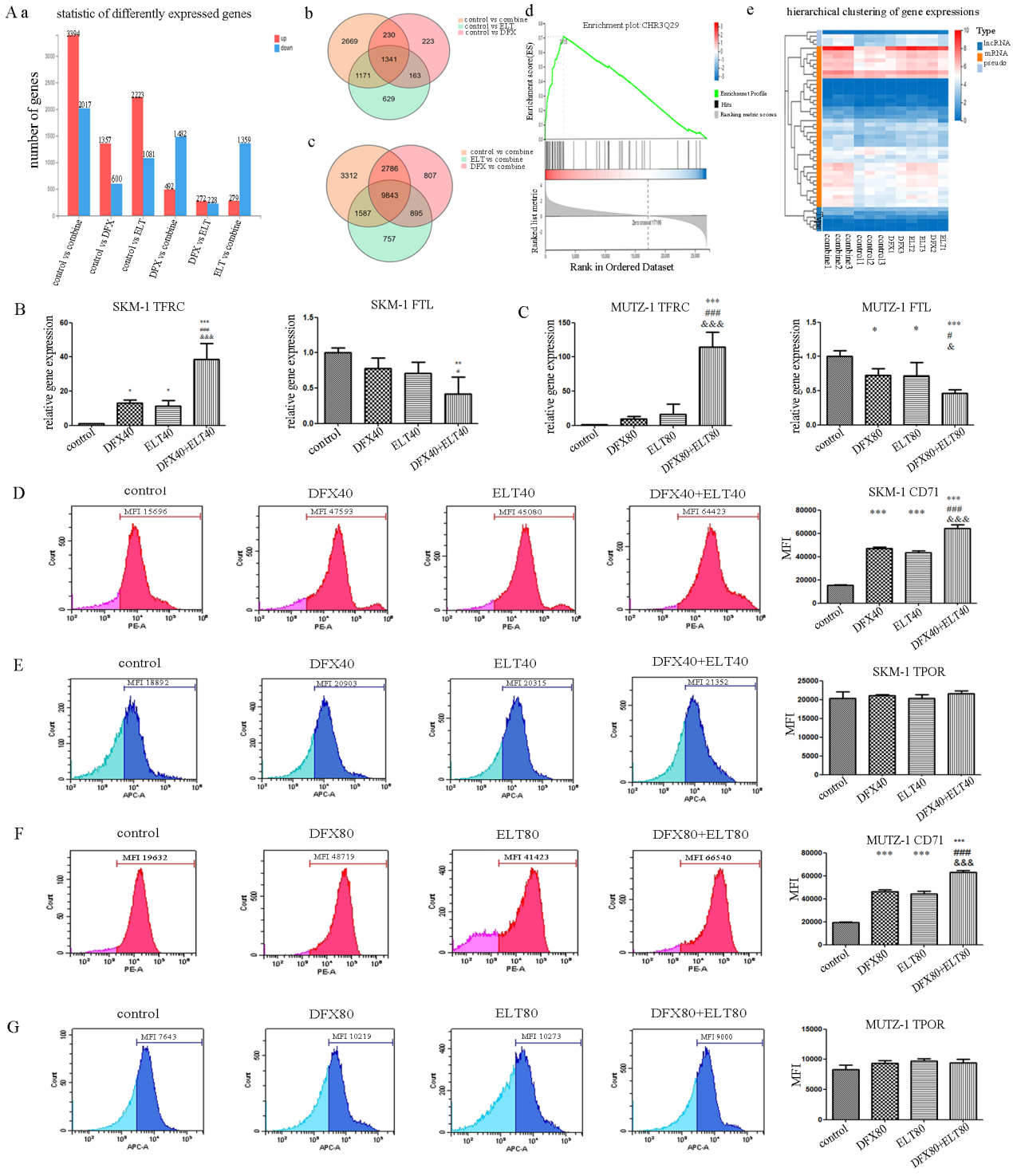

Figure 4 The altered iron metabolism after treatments with deferasirox (DFX) plus eltrombopag (ELT) in myelodysplastic syndrome (MDS) cells at 72 hours. (A) (a) RNA-sequence was performed on SKM-1 cells with DFX 40 μmol/L, ELT 40 μmol/L, or the two in combination at 72 hours. (b, c) Number of significantly different genes in each comparison group was shown. Compared with combination group, 1341 genes were upregulated, 9843 genes were downregulated. (d, e) GSEA functional gene analysis and differential gene expression analysis showed gene enrichments in iron metabolism–related signal pathway, which played important roles in inhibition of SKM-1 cells in combination group. (B, C) The iron metabolism–associated gene expression of TFRC was significantly upregulated in combination group, with significantly downregulated FTL. (D–G) The iron metabolism cell surface marker CD71 was significantly upregulated in the combination group, without affecting TPOR expression. Compared with the control group, * indicated $p<0.05$, ** indicated $p<0.01$, *** indicated $p<0.001$. Compared with the DFX group, # indicated $p<0.05$, ### indicated $p<0.001$. Compared with the ELT group, & indicated $p<0.05$, &&& indicated $p<0.001$.

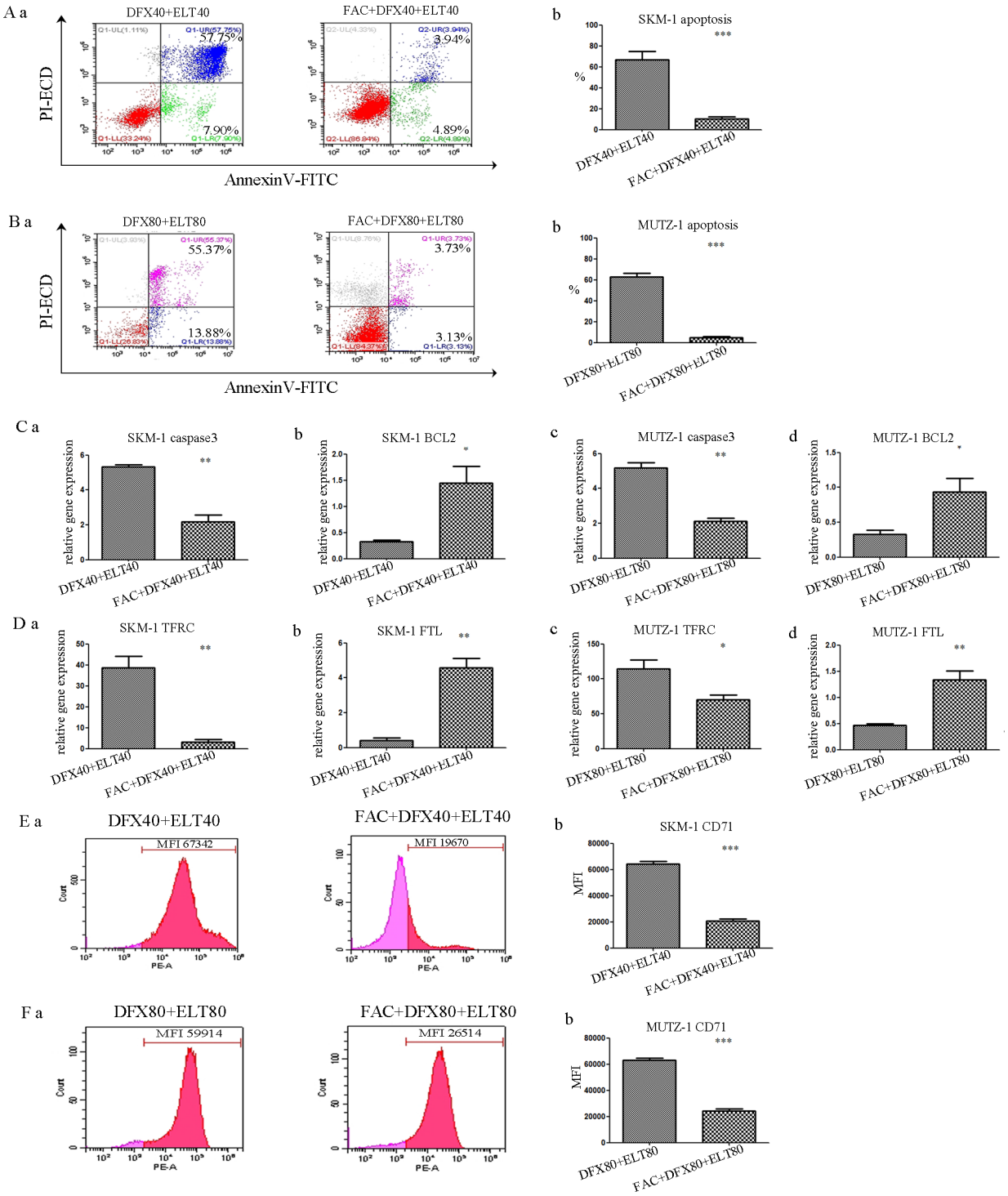

Figure 5 Iron rescue assay with ferric ammonium citrate (FAC) partially decreased the apoptosis induced by the combination of deferasirox (DFX) and eltrombopag (ELT) in myelodysplastic syndrome (MDS) cells at 72 hours. (A, B) The apoptosis of (DFX+ELT) group significantly decreased after treatment with FAC. (C) The gene expression of caspase-3 in (DFX+ELT) group significantly downregulated after treatment with FAC, with upregulated BCL-2. (D) The gene expression of TFRC in (DFX+ELT) group significantly downregulated after treatment with FAC, with upregulated FTL. (E–F) The iron metabolism cell surface marker CD71 on (DFX+ELT) group significantly downregulated after treatment with FAC. Compared with combination group, * indicated p<0.05, ** indicated p<0.01, *** indicated p<0.001.

patients treated with ELT obtained platelet response and reduced the risk of bleeding, without significant increased risk of leukemia transformation.[17] ELT could significantly reduce the ferritin level of aplastic patients through iron chelation effects.[18] Previous study has shown that ELT is a powerful iron chelator and enhances iron mobilization from

cardiomyocytes when combined with deferiprone, deferoxamine, or DFX.[19] Based on the limited use of DFX and the potential benefits of ELT in patients with MDS, we sought to investigate whether DFX combination with ELT might give a synergistic effect in treatment of high-risk MDS.

In our study, MDS cells were treated with DFX, ELT or combination for 48 or 72 hours. The CI <1 in both cell lines indicated the synergistic antiproliferation effects of DFX plus ELT. Previous study has shown that ELT could activate phosphorylation of AKT and ERK1/2.[20] We found that DFX plus ELT significantly inhibited the proliferation of MDS cells, along with downregulated AKT, suggesting that DFX plus ELT may inhibit the proliferation of MDS cells by inhibiting AKT.

In addition to the effects of DFX plus ELT on cell proliferation, we further studied the effects on cell cycle. We found that either DFX or ELT could block the cell cycle of MDS cells, and the combination had the most obvious effect at G0/G1 phase. Chang *et al*[21] showed that DFX could block cell cycle, induce cell apoptosis, and inhibit ERK phosphorylation of leukemia cells. Meanwhile, DFX could improve the transcriptional activity and protein stability of the p53 family, improve the OS of patients with MDS and delay the leukemia transformation course.[22] We also found that the protein level of P53 increased in combination group, with decreased Cyclin D1. TP53 encodes tumor suppressor proteins and regulates the expression of numerous target genes involved in cell cycle arrest, apoptosis, DNA repair, autophagy and metabolism regulation.[23] Cyclin D1 is involved in cell cycle conversion, cancer cells often have defects in G1/S phase, leading to unregulated growth and progression of tumors.[24] Shi *et al*[25] showed that ELT plus decitabine could exert synergistic antileukemia effects by blocking cell cycle. We concluded that DFX plus ELT might inhibit cell cycle at G0/G1 phase and reduce cell proliferation at S phase and G2 phase by upregulating P53 and downregulating Cyclin D1 in MDS cells.

Meanwhile, single DFX or ELT could induce apoptosis of MDS cells, and even higher in combination group. Previous study has shown that DFX combination with decitabine significantly increase the levels of reactive oxygen species (ROS) in leukemia cells and induce apoptosis.[26] ELT induces mitochondrial dysfunction in leukemia cells, triggers PARP cleavage and activates caspase cascade–induced leukemia cell apoptosis.[27] Consequently, we further detected the apoptosis-related signal pathway, we found that DFX combination with ELT significantly upregulated caspase-3 and downregulated BCL2.

The combination of DFX and ELT significantly inhibited proliferation, arrested cell cycle and induced apoptosis in MDS cells, which inspired us to study the potential synergistic mechanisms. To explore the synergistic mechanisms of DFX plus ELT in inhibiting MDS cells more effectively, we examined the gene expressions in SKM-1 treated with DFX, ELT or combination through RNA-sequence. According to the results of RNA-sequence, we found that DFX plus ELT synergistically increased iron metabolism–related gene expressions in SKM-1, TFRC gene expression changed most, so we speculated that TFRC might play an important role in combination group. We further detected the iron metabolism–related genes TFRC and FTL expressions in MDS cells. We found that the gene expression of TFRC significantly upregulated in combination group, with significantly downregulated FTL, which meant that the combination of DFX and ELT played synergistic effects by affecting the synthesis of ferritin, feedback increasing the gene expression of TFRC, which promote extracellular iron intake in MDS cells. Previous study has shown that DFX could reduce CD34 cell expression, increase the quality of mitochondria and the expression of erythrocyte surface marker CD71.[28] As we all know, ELT promotes the generation of platelet depending on the TPOR receptor on megakaryocyte. The expressions of TPOR and CD71 on the cell surface of MDS cells were detected by flow cytometry, we found that the protein expression of CD71 in combination group significantly increased, without affecting TPOR. Therefore, we concluded that DFX combination with ELT might play synergistic effects on MDS cells by reducing ferritin in MDS cells and feedback increasing CD71 protein expression on the surface of MDS cells, without affecting the role of ELT in promoting platelet generation.

We further confirmed the effects of iron metabolism in apoptosis induced by DFX plus ELT on MDS cells by adding FAC which could increase the extracellular iron. Our current study showed that FAC could reduce the apoptosis induced by the combination of DFX and ELT in both SKM-1 and MUTZ-1 cells, with significantly decreased gene expression of caspase-3 and increased BCL2. Meanwhile, the iron metabolism–related gene expression of TFRC, as well as the cell surface marker CD71, was significantly downregulated, with significantly upregulated FTL. Previous study has shown that ELT played anti-leukemia effect by inhibiting cell proliferation, blocking cell cycle, increasing cell differentiation by reducing iron in cells.[29] Our results showed that DFX plus ELT might reduce cell ferritin, inhibit cell proliferation, induce apoptosis and arrest cell cycle of MDS cells. And the iron rescue assay could partially reverse the effects of the combination on apoptosis of MDS cells.

In conclusion, our study demonstrated that DFX plus ELT synergistically induced apoptosis of MDS cells via enhancing iron deprivation, which upregulated gene and protein expressions of TFRC and caspase-3, and downregulated BCL2, without affecting TPOR, and iron rescue assay could partially reverse the iron deprivation–associated apoptosis of MDS cells. These findings may provide a strategy to develop a novel chemotherapy regimen for patients with MDS.

Contributors MT carried out the molecular genetic studies. ZL and CL participated in the cell culture and performed the statistical analysis. LH carried out the flow cytometry analysis and drafted the manuscript. RF conceived of the study, participated in its design and responsible for the overall content. LH and MT contributed equally to this work. All authors read and approved the final manuscript.

Funding This work was supported by the National Natural Science Foundation of China (Grant no. 81770110); National Natural Science Foundation Youth Project of China (Grant no. 81800123 and 81900131); Tianjin Natural Science Foundation (Key Project) (Grant no. 16JCZDJC35300); Tianjin Science and Technology Plan Project (Grant no. 20YFZCSY00060); Tianjin Municipal Natural Science Foundation Youth Project (Grant no. 18JCQNJC80400); Science and Technology Project of Tianjin Health Commission Youth Project (Grant no. QN20006) and the Scientific Research Project of Tianjin Education Commission (Grant nos. 2018KJ043, 2018KJ045 and 2017KJ195).

Competing interests None declared.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID iDs
Lei Huang http://orcid.org/0000-0003-3998-9867
Zhaoyun Liu http://orcid.org/0000-0002-2730-1562

REFERENCES

1 Carreau N, Tremblay D, Savona M, *et al*. Ironing out the details of iron overload in myelofibrosis: lessons from myelodysplastic syndromes. *Blood Rev* 2016;30:349–56.
2 Huang L, Fu R. [Research progress of characteristics and mechanisms of iron overload affecting bone marrow hematopoiesis]. *Zhonghua Xue Ye Xue Za Zhi* 2019;40:709–12.
3 Porter JB, de Witte T, Cappellini MD, *et al*. New insights into transfusion-related iron toxicity: implications for the oncologist. *Crit Rev Oncol Hematol* 2016;99:261–71.
4 Liu H, Yang N, Meng S, *et al*. Iron chelation therapy for myelodysplastic syndrome: a systematic review and meta-analysis. *Clin Exp Med* 2020;20:1–9.
5 Zhang J, Shi P, Liu J, *et al*. Efficacy and safety of iron chelator for transfusion-dependent patients with myelodysplastic syndrome: a meta-analysis. *Hematology* 2019;24:669–78.
6 Breccia M, Voso MT, Aloe Spiriti MA, *et al*. An increase in hemoglobin, platelets and white blood cells levels by iron chelation as single treatment in multitransfused patients with myelodysplastic syndromes: clinical evidences and possible biological mechanisms. *Ann Hematol* 2015;94:771–7.
7 Hoeks M, Yu G, Langemeijer S, *et al*. Impact of treatment with iron chelation therapy in patients with lower-risk myelodysplastic syndromes participating in the European MDS registry. *Haematologica* 2020;105:640–51.
8 Zeidan AM, Griffiths EA. To chelate or not to chelate in MDS: that is the question! *Blood Rev* 2018;32:368–77.
9 Erickson-Miller CL, Delorme E, Tian S-S, *et al*. Preclinical activity of eltrombopag (SB-497115), an oral, nonpeptide thrombopoietin receptor agonist. *Stem Cells* 2009;27:424–30.
10 Will B, Kawahara M, Luciano JP, *et al*. Effect of the nonpeptide thrombopoietin receptor agonist eltrombopag on bone marrow cells from patients with acute myeloid leukemia and myelodysplastic syndrome. *Blood* 2009;114:3899–908.
11 Swaminathan M, Borthakur G, Kadia TM, *et al*. A phase 2 clinical trial of eltrombopag for treatment of patients with myelodysplastic syndromes after hypomethylating-agent failure. *Leuk Lymphoma* 2019;60:2207–13.
12 Svensson T, Chowdhury O, Garelius H, *et al*. A pilot phase I dose finding safety study of the thrombopoietin-receptor agonist, eltrombopag, in patients with myelodysplastic syndrome treated with azacitidine. *Eur J Haematol* 2014;93:439–45.
13 Nakagawa T, Matozaki S. The SKM-1 leukemic cell line established from a patient with progression to myelomonocytic leukemia in myelodysplastic syndrome (MDS)-contribution to better understanding of MDS. *Leuk Lymphoma* 1995;17:335–9.
14 Steube KG, Gignac SM, Hu ZB, *et al*. In vitro culture studies of childhood myelodysplastic syndrome: establishment of the cell line MUTZ-1. *Leuk Lymphoma* 1997;25:345–63.
15 Shenoy N, Vallumsetla N, Rachmilewitz E, *et al*. Impact of iron overload and potential benefit from iron chelation in low-risk myelodysplastic syndrome. *Blood* 2014;124:873–81.
16 List AF, Baer MR, Steensma DP, *et al*. Deferasirox reduces serum ferritin and labile plasma iron in RBC transfusion-dependent patients with myelodysplastic syndrome. *J Clin Oncol* 2012;30:2134–9.
17 Oliva EN, Alati C, Santini V, *et al*. Eltrombopag versus placebo for low-risk myelodysplastic syndromes with thrombocytopenia (EQoL-MDS): phase 1 results of a single-blind, randomised, controlled, phase 2 superiority trial. *Lancet Haematol* 2017;4:e127–36.
18 Zhao Z, Sun Q, Sokoll LJ, *et al*. Eltrombopag mobilizes iron in patients with aplastic anemia. *Blood* 2018;131:2399–402.
19 Vlachodimitropoulou E, Chen Y-L, Garbowski M, *et al*. Eltrombopag: a powerful chelator of cellular or extracellular iron(III) alone or combined with a second chelator. *Blood* 2017;130:1923–33.
20 Di Buduo CA, Currao M, Pecci A, *et al*. Revealing eltrombopag's promotion of human megakaryopoiesis through AKT/ERK-dependent pathway activation. *Haematologica* 2016;101:1479–88.
21 Chang Y-C, Lo W-J, Huang Y-T, *et al*. Deferasirox has strong anti-leukemia activity but may antagonize theanti-leukemia effect of doxorubicin. *Leuk Lymphoma* 2017;58:2176–84.
22 Calabrese C, Panuzzo C, Stanga S, *et al*. Deferasirox-Dependent iron chelation enhances mitochondrial dysfunction and restores p53 signaling by stabilization of p53 family members in leukemic cells. *Int J Mol Sci* 2020;21:7674.
23 Monti P, Menichini P, Speciale A, *et al*. Heterogeneity of *TP53* Mutations and P53 Protein Residual Function in Cancer: Does It Matter? *Front Oncol* 2020;10:593383.
24 Luo J, Yan R, He X, *et al*. Constitutive activation of STAT3 and cyclin D1 overexpression contribute to proliferation, migration and invasion in gastric cancer cells. *Am J Transl Res* 2017;9:5671–7.
25 Shi M, Xu F, Yang X, *et al*. The synergistic antileukemic effects of eltrombopag and decitabine in myeloid leukemia cells. *Cancer Manag Res* 2019;11:8229–38.
26 Li N, Chen Q, Gu J, *et al*. Synergistic inhibitory effects of deferasirox in combination with decitabine on leukemia cell lines SKM-1, THP-1, and K-562. *Oncotarget* 2017;8:36517–30.
27 Kalota A, Selak MA, Garcia-Cid LA, *et al*. Eltrombopag modulates reactive oxygen species and decreases acute myeloid leukemia cell survival. *PLoS One* 2015;10:e0126691.
28 Tataranni T, Agriesti F, Mazzoccoli C, *et al*. The iron chelator deferasirox affects redox signalling in haematopoietic stem/progenitor cells. *Br J Haematol* 2015;170:236–46.
29 Roth M, Will B, Simkin G, *et al*. Eltrombopag inhibits the proliferation of leukemia cells via reduction of intracellular iron and induction of differentiation. *Blood* 2012;120:386–94.