Anti-leukemic properties of deferasirox via apoptosis in murine leukemia cell lines

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Background
Although deferasirox (DFX) is reported to have anti-tumor effects, its anti-leukemic activity remains unclear. We evaluated the effect of DFX treatment on two murine lymphoid leukemia cell lines, and clarified the mechanisms underlying its potential anti-leukemic activity.

Methods
L1210 and A20 murine lymphoid leukemia cell lines were treated with DFX. Cell viability and apoptosis were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and fluorescence-activated cell sorting (FACS) analysis, respectively. Immunoblotting was performed to detect the expression of key apoptotic proteins.

Results
In dose- and time-dependent manner, DFX decreased viability and increased apoptosis of murine leukemic cells. Fas expression was significantly higher in A20 cells than in L1210 cells at all DFX concentrations tested. Although both cell lines exhibited high caspase 3 and caspase 9 expression, a critical component of the intrinsic mitochondrial apoptotic pathway, expression was greater in L1210 cells. In contrast, caspase 8, a key factor in the extrinsic apoptotic pathway, showed greater expression in A20 cells. Cytochrome c expression was significantly higher in L1210 cells. In both cell lines, co-treatment with ferric chloride and DFX diminished the expression of these intracellular proteins, as compared to DFX treatment alone.

Conclusion
Treatment with DFX increased caspase-dependent apoptosis in two murine lymphoid leukemia cell lines, with differing apoptotic mechanisms in each cell line.

Key Words
Lymphoid leukemia, Deferasirox, Apoptosis, Caspase

INTRODUCTION

Hematological malignancies such as acute leukemia require frequent blood transfusions [1]. Multiple blood transfusions may lead to secondary iron overload (SIO), which promotes cancer cell growth and induces deleterious effects on the innate immune system [2-4]. An iron-chelating agent (ICA) is used to minimize the adverse effects of iron overload in patients with SIO who require repeated blood transfusions [5, 6].

Deferoxamine (DFO) and deferasirox (DFX) are ICAs with different iron-chelating mechanisms. DFO has a short half-life, and must be injected intravenously or subcutaneously, because it is a large and highly hydrophilic molecule [7, 8]. In contrast, DFX has relatively high lipophilicity and is an orally administered iron chelator that was developed to treat transfusional iron overload. Previous work indicates that DFX is safe and effective for the reduction of iron overload [8, 9].

Various malignant cells have been used to study the anti-tumor effect of ICAs [10, 11]. Messa et al. demonstrated that DFX exerts anti-leukemic effects in a myeloid leukemia cell line by inhibiting nuclear factor-κB (NF-κB) activity.
Cell lines, cell culture, and DFX treatment

Cells (10^5 cells/well) were seeded in flat-bottom plates and maintained in an incubator (5% CO₂/95% air) at 37°C. After 24 hr incubation, cells were treated with 50 μM DFX with or without 20 μM FeCl₃ for 0, 6, 12, 24, and 48 hr. The cells were collected, washed twice with cold PBS, and lysed in radioimmunoprecipitation assay buffer (Thermo Scientific, Rockford, IL, USA) containing a mixture of protease inhibitors (Thermo Scientific) for 15 min on ice. After centrifugation, the supernatants underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were blotted onto a membrane (Whatman GmbH, Dassel, Germany) and blocked with 5% skim milk (BD Sciences) for 30 min at room temperature. The membranes were incubated overnight at 4°C with primary antibodies. The proteins were analyzed by western blot using anti-caspase-3 (Abcam, Cambridge, MA, USA), anti-caspase-9 (Novus Biologicals, LLC., Wilmington, DE, USA), anti-caspase-8 (Abcam), anti-β-actin (Abcam), and anti-poly ADP ribose polymerase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with 1× Tris-buffered saline with Tween 20 (Cell Signaling Technology, Beverly, MA, USA) three times for 10 min, the proteins were probed at room temperature for 2 hr with anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology). Immunoreactive proteins were detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and the Western Blotting Detection System (Biomics, Hayward, CA, USA).

Immunoblotting analysis

L1210 and A20 cells were seeded in a 6-well plate (1×10⁶ cells/mL) and maintained in an incubator (5% CO₂/95% air) at 37°C for 24 hr. The cells were treated with 50 μM DFX with or without 20 μM FeCl₃ for 0, 6, 12, 24, and 48 hr. The cells were collected, washed twice with cold PBS, and lysed in radioimmunoprecipitation assay buffer (Thermo Scientific, Rockford, IL, USA) containing a mixture of protease inhibitors (Thermo Scientific) for 15 min on ice. After centrifugation, the supernatants underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were blotted onto a membrane (Whatman GmbH, Dassel, Germany) and blocked with 5% skim milk (BD Sciences) for 30 min at room temperature. The membranes were incubated overnight at 4°C with primary antibodies. The proteins were analyzed by western blot using anti-caspase-3 (Abcam, Cambridge, MA, USA), anti-caspase-9 (Novus Biologicals, LLC., Wilmington, DE, USA), anti-caspase-8 (Abcam), anti-β-actin (Abcam), and anti-poly ADP ribose polymerase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with 1× Tris-buffered saline with Tween 20 (Cell Signaling Technology, Beverly, MA, USA) three times for 10 min, the proteins were probed at room temperature for 2 hr with anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology). Immunoreactive proteins were detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and the Western Blotting Detection System (Biomics, Hayward, CA, USA). Mitochondria were isolated for the cytochrome assay using a Qproteome Mitochondria Isolation kit (Qiagen, Hilden, Germany). The two cell lines were probed with anti-cytochrome c (Abcam), and the Chemi-DOC system (Biomics) was used to detect signals.

Statistical analysis

Data are presented as the mean±standard deviation of three independent experiments. Statistical analyses were per-
formed using GraphPad Prism ver. 5.0 software (GraphPad Software, San Diego, CA, USA). Comparisons among the groups were performed by analysis of variance with SPSS ver. 12.0 for Windows (SPSS Inc., Chicago, IL, USA). A $P$ value $<0.05$ was considered significant.

## RESULTS

### Effect of DFX on murine leukemia cell viability

The viability of both L1210 and A20 cells decreased upon DFX exposure (Fig. 1). Longer treatment (48 hr vs. 24 hr) at higher DFX concentrations resulted in a greater decrease in cell viability. Of note, cell viability in L1210 cells decreased at lower DFX concentrations than A20 cells, in which a significant decrease in viability was only observed when treated with 50 $\mu$M DFX for 48 hr. The addition of FeCl$_3$ preserved cell viability in the presence of 12.5 $\mu$M DFX for 24 and 48 hr in L1210 cells, and 50 $\mu$M DFX for 48 hr in A20 cells.

### Effect of DFX on murine leukemia cell apoptosis

Apoptosis assays were performed to clarify whether increased cell apoptosis induced decreased cell viability following DFX treatment. The assays revealed an increase in apoptosis after DFX treatment in both murine leukemia cell lines (Fig. 2). In accordance with the proliferation assay results, lengthier treatment (48 hr vs. 24 hr) at higher DFX concentrations resulted in a greater percentage of apoptotic cells in both cell lines. As with the cell viability assay, the addition of FeCl$_3$ significantly decreased apoptosis at certain DFX concentrations (Fig. 2).

### Effect of DFX on Fas expression in murine leukemia cells

Both leukemia cell lines were analyzed for CD95 (Fas) expression after DFX treatment to characterize the mechanisms regulating DFX-mediated cell apoptosis. CD95 expression increased in both L1210 and A20 cells upon treatment with DFX. Of note, A20 cells displayed a significantly greater percentage of CD95-expressing cells than the L1210 cell line for all DFX concentrations, regardless of FeCl$_3$ addition (Fig. 3).

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**Fig. 1.** Viability of L1210 (A, B) and A20 cells (C, D) after DFX treatment with or without FeCl$_3$, according to treatment time (24 vs. 48 hr) and DFX concentration (3.125–75 $\mu$M). Experiments were performed in triplicate. All data are presented as the mean ± standard error ($^aP < 0.05$, $^bP < 0.01$).
Fig. 2. Measurement of apoptosis in L1210 (A, B) and A20 cells (C, D) after DFX treatment with or without FeCl₃, according to treatment time (24 vs. 48 hr) and DFX concentration (12.5–50 μM). Experiments were performed in triplicate. All data are presented as the mean±standard error (a) \( P < 0.05 \), (b) \( P < 0.01 \).

Fig. 3. Measurement of CD95 (Fas) expression in L1210 and A20 cells after 24 h DFX treatment with or without FeCl₃, according to DFX concentration (12.5–50 μM). Experiments were performed in triplicate. All data are presented as the mean±standard error (a) \( P < 0.05 \), (b) \( P < 0.01 \).
Expression of apoptotic proteins in DFX-treated murine leukemia cells

The expression of apoptotic proteins including caspases, poly ADP ribose polymerase (PARP), and Bax proteins was determined after treating the leukemia cell lines with 50 μM DFX at sequential time periods up to 48 hr treatment. Caspase 3 expression increased upon prolonged DFX treatment in both cell lines, with peak expression observed after 48 hr of treatment (Fig. 4). Caspase 9, which is predominantly expressed in the intrinsic mitochondrial apoptotic pathway, was significantly expressed only in the L1210 cell line, whereas caspase 8, a key protein in the extrinsic apoptotic pathway, was highly expressed in A20 cells. Expression of caspases 3, 8, and 9 was stronger in leukemia cells treated with DFX alone, as compared to cells treated with both DFX and FeCl3. PARP expression, which was measured to verify the apoptotic pathway, was confirmed in both leukemia cell lines, whereas Bax, a marker of the mitochondrial apoptotic pathway, was weakly expressed in both cell lines.

Cytochrome c release in murine leukemia cells treated with DFX

Expression of cytochrome c was measured in both leukemia cell lines after treatment with 50 μM DFX to further...
SIO due to repeated blood transfusion affects heart, liver, and other vital organ function [14]. ICAs have been used to treat transfusional iron overload and overcome the complications of SIO, and are considered potential anti-cancer agents [15, 16]. Several studies support the anti-leukemic effects of ICAs [3, 12, 13, 17-20]. This was based on evidence of anti-tumor effects, including the inhibition of proliferation and differentiation via inhibited DNA synthesis and apoptosis induction through caspase 3 and 9 [18-20]. The effects of DFX on human esophageal cancer and hepatoma cell line viability (OE33, OE19, and OE21) have been reported [21, 22]. Ford et al. demonstrated that DFX decreases esophageal cancer cell viability and proliferation [21]. In addition, Gaboriau et al. reported increased hepatoma cell viability in the presence of iron after ICA treatment [22].

Our results showed that the cell viability of two murine lymphoid leukemia cell lines, L1210 and A20, decreased in a concentration- and time-dependent manner. Of note, the L1210 cells showed decreased viability at lower DFX concentrations when compared to A20 cells, possibly indicating greater sensitivity to the effects of DFX.

The apoptosis assay also revealed a time- and concentration-dependent increase in apoptosis following DFX treatment, indicating that DFX-mediated apoptosis was responsible for decreased cell viability. Our results are similar to those of ICA-treated Jurkat cells and malignant lymphoma cell lines undergoing apoptosis [23, 24].

Whether the anti-tumor activity of DFX is linked to iron depletion via iron chelation is not clear, as several previous studies concluded that this activity is either independent of the effects of iron chelation or clearly dependent on iron chelation [12, 25]. In our study, we found that the addition of FeCl₃ significantly lowered DFX-mediated apoptosis in certain scenarios, particularly with high-dose, prolonged DFX treatment. However, the cell salvaging potential of FeCl₃ was not uniform through all experiments, indicating that iron chelation may have a partial role in cell death, but does not account for the entire mechanism of action.

Expression of CD95 (Fas) was significantly higher in both leukemia cell lines upon DFX treatment. However, CD95 expression was significantly higher in A20 cells than L1210 cells at all DFX concentrations tested, indicating that CD95-mediated apoptosis may play a greater role in A20 cells.

We determined the expression of critical apoptotic proteins to clarify the mechanisms regulating apoptosis in each cell line. Caspase 3 expression was increased in both cell lines following prolonged DFX exposure, indicating that apoptosis occurred through a caspase-dependent pathway. However, caspase 9 and 8 expression differed between the cell lines, with L1210 cells displaying significantly higher caspase 9 expression and A20 cells showing higher caspase 8 expression. As caspase 9 is a key component of the intrinsic mitochondrial apoptotic pathway, our results indicate that this pathway may be the predominant mode of L1210 cell apoptosis. In contrast, caspase 8 is a critical protein in the extrinsic apoptotic pathway, which may be the key mechanism of A20 cell apoptosis. Supporting these results, expression of cytochrome c, a mitochondrial protein regulating the intrinsic apoptotic pathway, was significantly higher in L1210 cells. Mechanisms of DFX-mediated apoptosis have previously been reported for other malignancies. Caspase-dependent apoptosis was also observed in DFX-treated myeloid leukemia cells [19]. Caspase 3/7-, caspase 9-, and Bax-induced apoptosis was observed in a malignant lymphoma cell line [24]. Messa et al. demonstrated that oral iron chelation therapy has anti-tumor effects via NF-κB inhibition in patients with myelodysplastic syndrome [12].

Importantly, we found that the addition of FeCl₃ decreased the expression of caspases and cytochrome c in both cell lines, underscoring the notion that DFX-mediated cell apoptosis occurs in the context of iron deprivation. However, the lack of a uniform effect of FeCl₃ dictates that further studies are necessary to conclude whether the anti-tumor activity of DFX is dependent on its iron-chelating ability.

In conclusion, treatment with DFX increased apoptosis in two murine lymphoid leukemia cell lines through caspase-dependent pathways. The dominant apoptotic pathway, however, differed between cell lines, with the intrinsic mitochondrial pathway having a key role in L1210 cells, while the extrinsic pathway was more significant in A20 cells. Thus, the exact apoptotic mechanism may differ between lymphoid leukemia cell lines, although caspase-dependent apoptosis may be a common feature. Our results add to previous literature on the anti-tumor activity of DFX and underscore the mechanism of action. Whether this apoptotic activity is dependent on iron chelation requires further study.

**Authors’ Disclosures of Potential Conflicts of Interest**

No potential conflicts of interest relevant to this article were reported.

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