DEFERASIROX DEPLETES Bcl-xL AND PROMOTES IRON DEPLETION-DEPENDENT DEATH OF PROXIMAL TUBULAR CELLS

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INTRODUCTION:

Deferasirox (also known as ICL670) is a potent specific N-substituted bis-hydroxyphenyl-triazole class oral tridentate iron chelator in clinical use since 2005 as first-line therapy for blood transfusion-related iron overload.

This drug can result in an acute or chronic decrease in glomerular filtration rate (GFR) and/or features of proximal tubular dysfunction. Deferasirox promotes apoptosis of cultured proximal tubular cells, but the molecular triggers and therapeutic implications have not been well characterized.

Besides higher Deferasirox accumulation, proximal tubular cells could be more sensitive to Deferasirox toxicity because they contain many mitochondria, which provide energy for transport processes. Mitochondria are key regulators of intracellular iron homeostasis and key mitochondrial proteins require iron for correct functioning.

AIM:

Explore the molecular mechanisms of Deferasirox nephrotoxicity in proximal cultured tubular cells as well as the opportunities for therapeutic manipulation.

METHODS:

Cells and reagents:

MCT cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY), 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin and 100µg/mL streptomycin, in 5% CO2 at 37°C. A MCT cell line overexpressing BclxL has been obtained in our laboratory. Deferasirox and deferasirox-iron complex (Santa Cruz Biotechnology, Santa Cruz, CA) were dissolved in DMSO and methanol respectively. Benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) (BD Bioscience, San Jose, CA, USA) and necrostatin-1 (SIGMA, St. Louis, MO, USA) were dissolved in DMSO and added 1hour prior to stimuli.

Assessment of cell death:

Cell viability was estimated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma- Aldrich) colorimetric assay.

Cell death were analyzed by annexin V/7-AAD staining and cells counted by flow cytometry using BD FACS Diva Software (BD Biosciences). Cell morphology following deferasirox treatment was examined by transmission electronic microscopy.

Mitochondrial membrane potential (MMP):

MMP were determined by tetramethylrhodamine methyl ester (TMRM) fluorescence (Molecular Probes, Lifetechnologies). Fluorescence intensity was measured by flow cytometry using BD FACS Diva Software (BD Bioscience).

Caspase 3 Activity:

Caspase 3 Activity was measured following the manufacturer's instructions of Caspase-3/CPP32 Colorimetric Assay Kit (MLB).

Immunostaining:

Cells were incubated with anti-TOMM22 (1:100, Abcam), anti-cytocrome C (1:500, BDPharmigen) or anti-BAX (1:100 Calbiochem) followed by Alexa488 or Alexa562 conjugated secondary antibody respectively (1:300, Invitrogen).

Western Blot:

Samples homogenized in lysis buffer were separated by 10-12% SDS-PAGE under reducing conditions. Primary antibodies were anti-Bax (1:100 Calbiochem), anti-BclxL (1:250, Santa Cruz Biotechnology) and anti-cytochrome C (1:250, BDPharmigen). Blots were then probed with anti-α-tubulin (1:2000, Sigma) or anti-cytochrome oxidase subunit IV (1:200, Santa Cruz, Biotechnology) and levels of expression were corrected for minor differences in loading.

siRNA transfection:

Cells were seeded and transfected on the following day with 20nM of a scrambled siRNA or siRNA against RIP3 by using lipofectamine reagent (Invitrogen).

Statistics:

Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as mean ± SD. Significance at the $p<0.05$ level was assessed by Student´s t test for two groups of data and ANOVA for three of more groups.

CONCLUSIONS:

Deferasirox is directly toxic over cultured proximal tubular cells, induces mitochondrial dysfunction and cell death cannot be rescued by commonly used apoptosis and necroptosis inhibitors. BclxL overexpression was protective, further pointing out to mitochondria as sites of injury.

Deferasirox provides a new and challenging model of proximal tubular cell death that may provide new clues to the molecular mechanism of nephrotoxicity and the adverse consequences of iron depletion.

RESULTS:

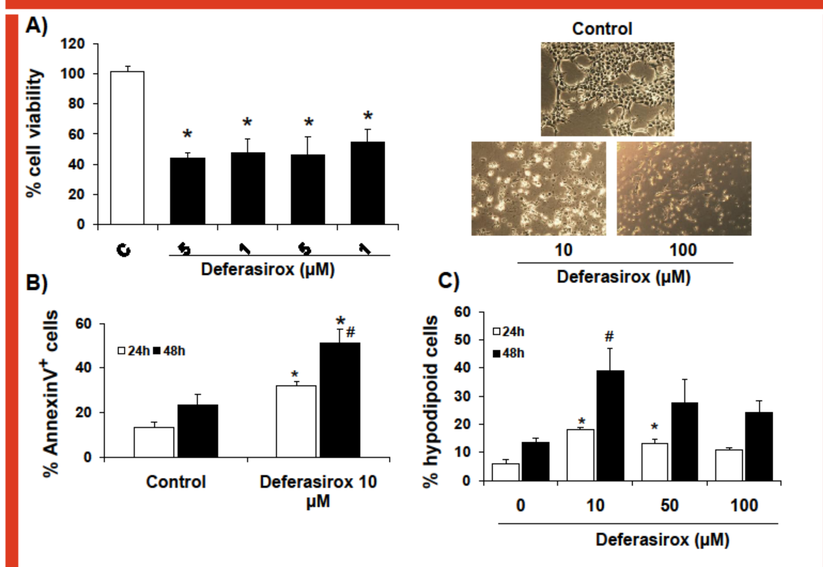

Figure 1. Deferasirox induces death of proximal tubular epithelial cells. A) Tubular cell viability at 24h by MTT assay and contrast phase microscopy photographs. **B)** Time course of annexin V staining in tubular cells. **C)** Deferasirox increased the percentage of hypodiploid cells.

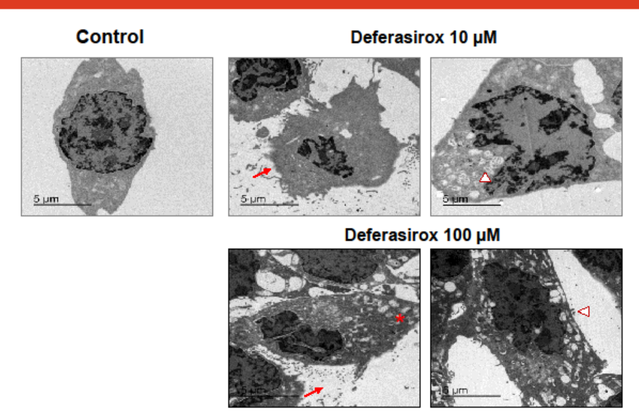

Figure 2. Morphology of murine tubular cells treated with deferasirox. TEM of cells exposed to deferasirox disclosed a typical necrotic morphology, characterized by membrane rupture (arrows), extensive vacuolization (arrowsheads), and, destroyed mitochondria (asterisks) (x5000).

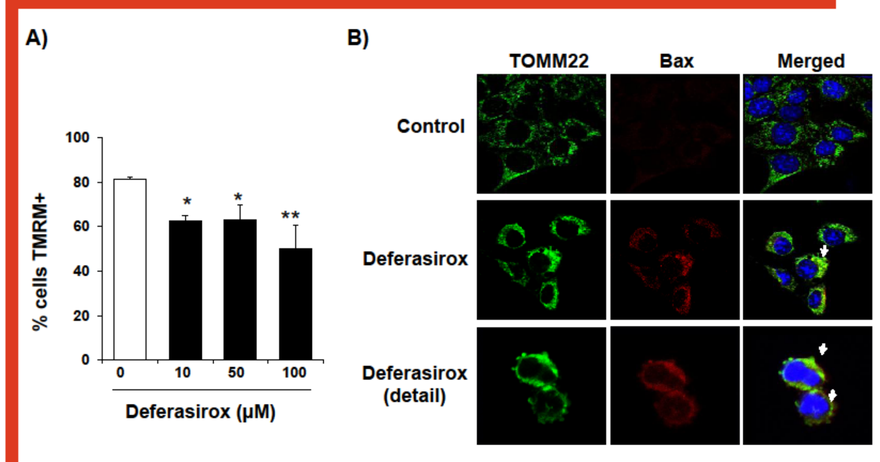

Figure 3. Deferasirox induces mitochondrial stress in tubular cells. A) Loss mitochondrial membrane potential (MMP) was assessed by TMRM. **B)** Immunofluorescence of TOMM22 and Bax. **C)** Cytochrome C is released from mitochondria in tubular cells exposed to deferasirox in a time-dependent fashion.

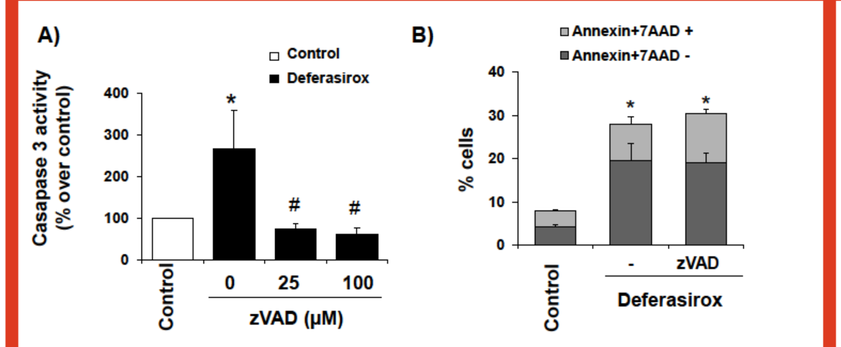

Figure 4. Caspase inhibition prevents caspase-3 activation but does not increase viability of tubular cells exposed to deferasirox. A) Pretreatment with the zVAD prevented caspase-3 activation. **B)** Cell death induced by deferasirox is not prevented by zVAD.

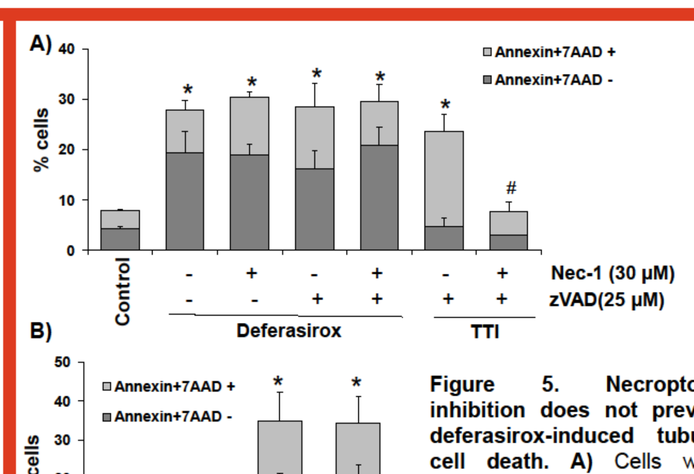

Figure 5. Necroptosis inhibition does not prevent deferasirox-induced tubular cell death. A) Cells were pretreated with zVAD and/or Nec-1 before exposure to Deferasirox. **B)** RIP3 was targeted with siRNA, and cells were exposed deferasirox.

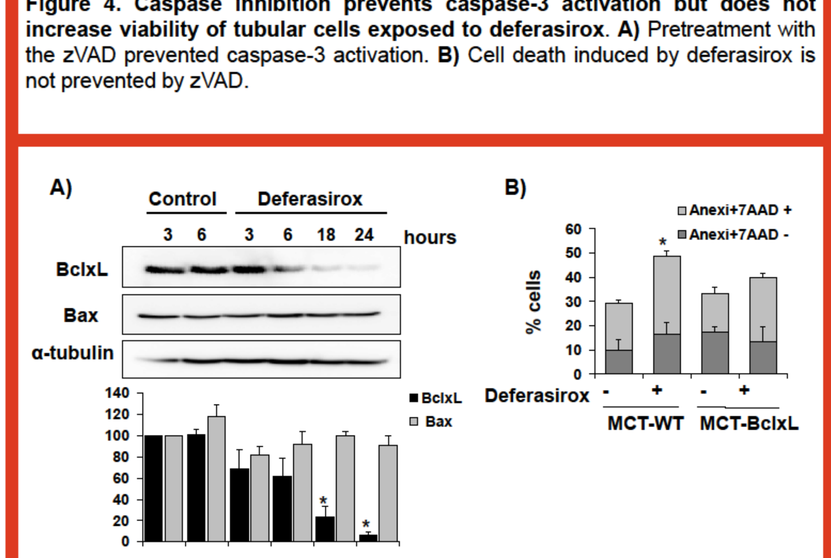

Figure 6. BclxL downregulation is involved in deferasirox induced cell death. A) BclxL and Bax protein expression in tubular cells exposed to 10µM deferasirox for different times. **B)** BclxL-overexpressing cells were protected from cell death induced by deferasirox.

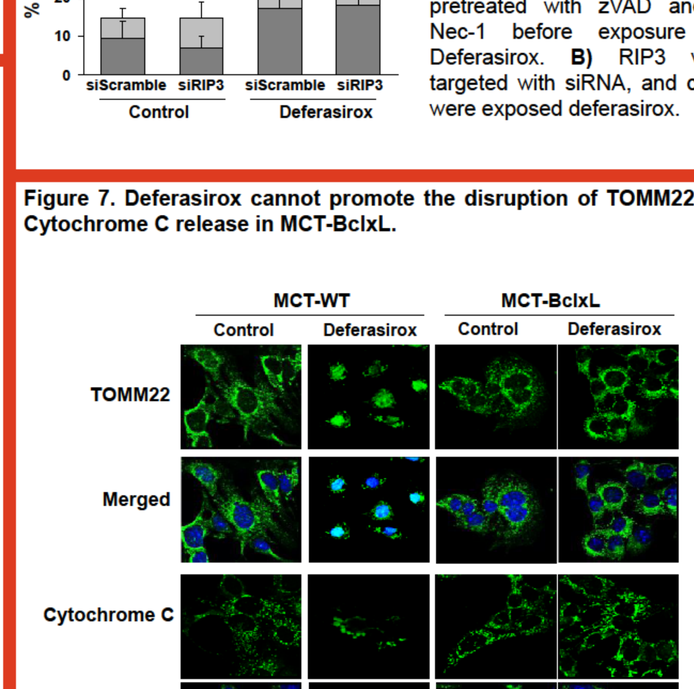

Figure 7. Deferasirox cannot promote the disruption of TOMM22 and Cytochrome C release in MCT-BclxL.

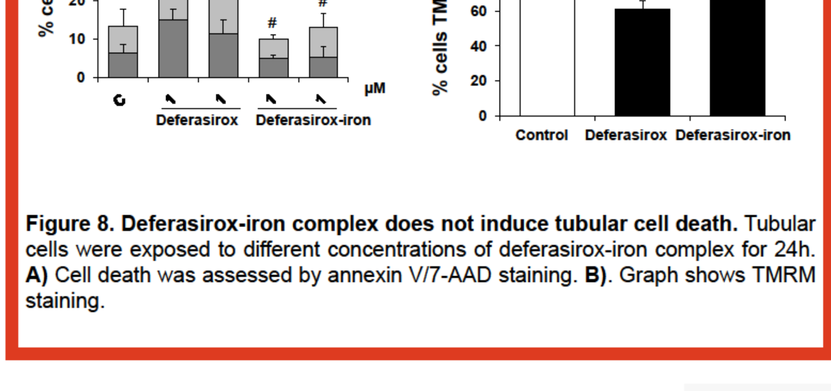

Figure 8. Deferasirox-iron complex does not induce tubular cell death. Tubular cells were exposed to different concentrations of deferasirox-iron complex for 24h. **A)** Cell death was assessed by annexin V/7-AAD staining. **B)**. Graph shows TMRM staining.

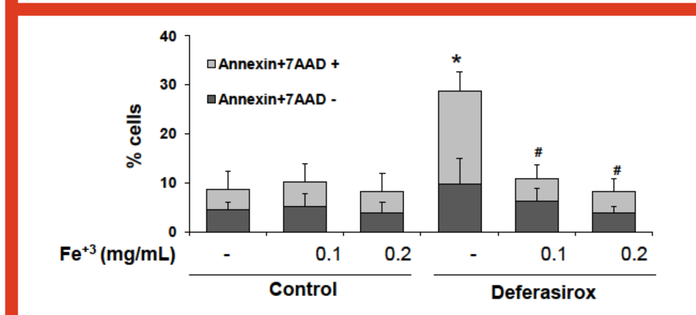

Figure 9. Iron-loading prevented deferasirox-induced tubular cell death. Flow cytometry of annexin V/7-AAD staining.

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