IN VIVO CHARACTERIZATION OF EED INHIBITORS, NOVEL SMALL MOLECULE FETAL HEMOGLOBIN INDUCERS FOR SICKLE CELL DISEASE

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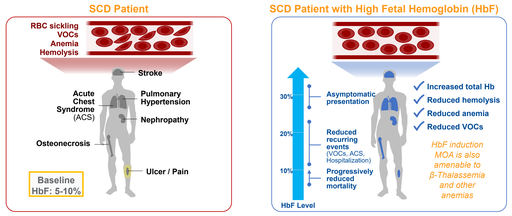
INTRODUCTION

Fetal Hemoglobin (HbF) Mitigates Mortality and Morbidity Risks Associated with Sickle Cell Disease



People living with SCD can have additional mutations that cause a condition known as hereditary persistence of fetal hemoglobin (HPFH), which leads to reduced or no symptoms in patients with SCD and β-thalassemia

Increased HbF levels over typical baseline measurements relieve severity of symptoms like hemolysis, anemia and VOCs in people living with Sickle Cell Disease

FulcrumSeek Identified Embryonic Ectoderm Development (EED) as a Target for HbF Induction

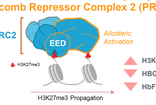
CRISPR + Compound Screening Engine
Experimentally screened candidate targets

Computational Data Mining
Computationally mined candidate targets

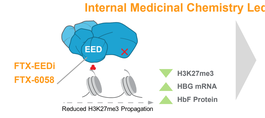
Identified Targets that Regulate HbF

PRC2, BCL11A, NuRD, HDACs, LSD1, DNMT1, IKZF1, IKZF3, SPOP

Identified EED as a Novel Drug Target of Polycomb Repressor Complex 2 (PRC2)



Internal Medicinal Chemistry Led to Potent and Selective EED Inhibitors



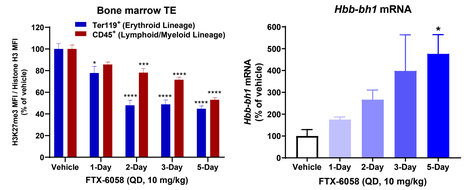
FTX-6058 - EED K_D = 0.163 nM / PRC2 IC_{50} < 5 nM
FTX-EEDi - EED K_D = 0.305 nM / PRC2 IC_{50} < 5 nM

Highly Selective
Clean Off-target Profile
Fulcrum Therapeutics is developing FTX-6058 in the clinic for Sickle Cell Disease

Inhibition of EED leads to induction of HbF protein through direct reduction of H3K27me3

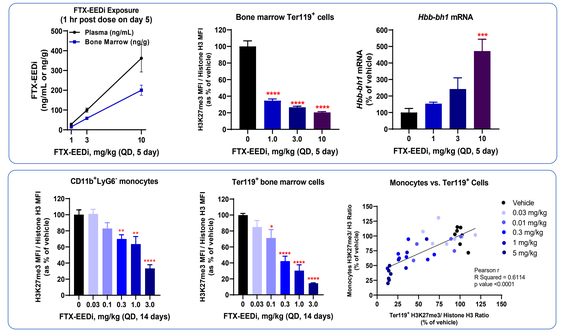
RESULTS

Target Engagement of FTX-6058 in Wildtype Mice



Chronic Dosing of FTX-6058 significantly decreases our target engagement biomarker and increases Hbb-bh1 mRNA 2-3-fold over vehicle

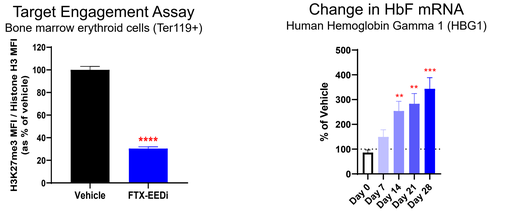
Target Engagement in Bone Marrow and Monocytes



Peripheral blood target engagement correlates with bone marrow target engagement which suggests it could be used as a translatable biomarker in the clinic

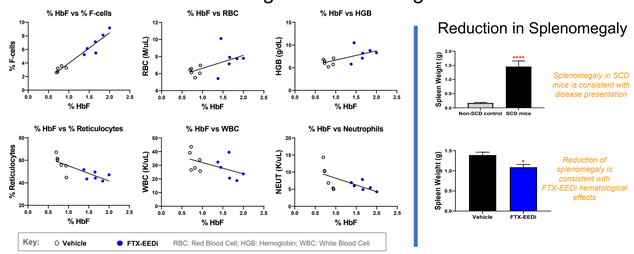
FTX-EEDi and FTX-6058 Demonstrated Robust Target Engagement and Hematological Effects When Dosed in the Townes Mouse Model of Sickle Cell Disease

Robust Target Engagement and Increases in HBG1 mRNA



Maximal target engagement maintains ~30% of H3K27me3 mark
Chronic FTX-EEDi dosing increases HbF mRNA (HBG1) 3-fold over time

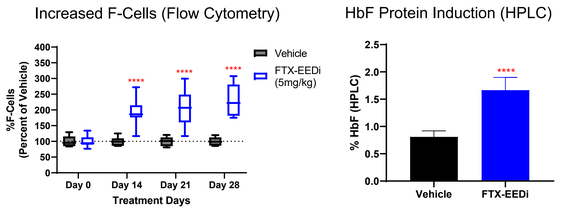
FTX-EEDi-Induced Increases in HbF Correlate with Changes in Hematological Parameters



Correlation with RBC, HGB and % Reticulocytes suggest reduction in hemolysis induced anemia
Correlations with WBC and Neutrophils suggest reduction of inflammatory response

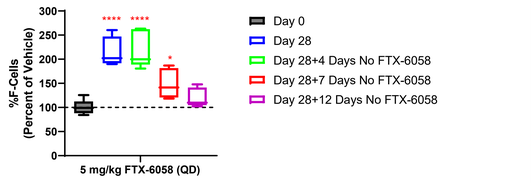
Reduction in Splenomegaly

Increases in HBG1 mRNA is Translating to Increased %F-Cells and Fetal Hemoglobin Protein



2-3-fold induction in %F-Cells and HbF Protein

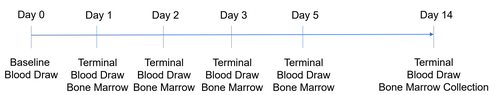
Persistent F-Cell Increases Following FTX-6058 Dosing Cessation at Day 28



FTX-6058 demonstrates time-dependent increases in F-cell and HbF expression
Consistent with MOA and RBC half-life, F-cell increases demonstrate robust persistence, with no loss of effect up to 4 days after dosing cessation

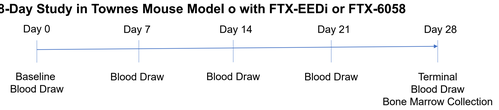
METHODS

1-, 2, 3-, 5- and 14- Day Study in CD-1 Mice with FTX-6058 and FTX-EEDi



- CD-1 mice were 8 weeks of age at the start of the study
- CD-1 mice received FTX-6058 (10 mg/kg), FTX-EEDi (5 mg/kg) or vehicle, PO, QD. Dose was chosen based on activity in *in vitro* assays.
- Terminal samples collected 1-hour after last dose.

28-Day Study in Townes Mouse Model o with FTX-EEDi or FTX-6058



- Townes SCD mice were 8 weeks of age at the start of the study to model a severe disease phenotype.
- Townes SCD mice received FTX-6058 (5 mg/kg), FTX-EEDi (5 mg/kg) or vehicle, PO, QD. Dose was chosen based on activity in *in vitro* assays.
- Interim bleeds collected before daily dosing.
- Terminal samples collected 1-hour after last dose.
- For FTX-6058 in Townes SCD mice, a satellite group had blood drawn 4-, 7-, 12-days post last FTX-6058 dose

For all flow cytometric methods, the Cytek Aurora cytometer was used for data acquisition. Spectral unmixing was preformed using Cytek's SpectroFlo software. Post unmixing analysis was done using FlowJo (Becton Dickinson & Company) software.

% F Cells
7.5µL whole blood was used per test. The RBCs were fixed in ice cold 0.05% glutaraldehyde and subsequently permeabilized in ice cold 0.1% Triton X-100. Fetal hemoglobin staining was accomplished using Fetal Hemoglobin mAb Test Kit, R-PE Conjugate (ThermoFisher # HFH04). For analysis, 2 tests were set up per sample, 1) a fluorescence minus one (FMO) and 2) Fetal Hemoglobin mAb. The positive and negative boundary for fetal hemoglobin staining was established using the FMO.

Ter119 BM Cells/ CD45 cells
The bone marrow was filtered, washed, and counted. 1.5e6 cells were used per test. For cellular subletting, the cells were stained with anti-CD45 BUV395 (BD Biosciences # 564279) and anti Ter119 Alexa Fluor 488 (Biolegend # 108417). The fixable viability day Zombie NIR (Biolegend # 423106) was added to the surface stain cocktail. The cells were washed three times. After the final wash, fixation, lysing, and permeabilization was carried out using the PerFix EXPOSE kit (Beckman Coulter # B26976) following the manufacturer's protocol for isolated cells. Staining for H3K27me3 and total histone H3 was accomplished using anti Tri-Methyl-Histone H3 (Lys27) Alexa Fluor 647 (Cell Signaling Technology # 12158S) and anti Pan Histone Pacific Blue (Cell Signaling Technology # 12167S), respectively. Cells were washed three times before acquisition on the Aurora cytometer.

CD11b monocytes
EDTA was used as the anticoagulant. 100µL whole blood in was used per test. For cellular subletting, the cells were stained with a cocktail of anti CD45 PE/Cyanine 7 (ThermoFisher # 25-0451-82), anti Ly-6G (Gr-1) PE/eFluor610 (ThermoFisher # 61-5931-82), anti CD3 Super Bright 702 (ThermoFisher # 67-0032-82), and anti CD11b PE (ThermoFisher # 12-0112-82). The fixable viability day Zombie NIR (Biolegend # 423106) was added to the surface stain cocktail. The cells were washed once. After the wash, fixation, lysing, and permeabilization was carried out using the PerFix EXPOSE kit (Beckman Coulter # B26976) following the manufacturer's protocol for whole blood. Staining for H3K27me3 and total histone H3 was accomplished using anti Tri-Methyl-Histone H3 (Lys27) Alexa Fluor 647 (Cell Signaling Technology # 12158S) and anti Pan Histone Pacific Blue (Cell Signaling Technology # 12167S), respectively. Cells were washed three times before acquisition on the Aurora cytometer.

%Hbf Induction
Assay was conducted at Spectrus (Beverly, MA). HbF induction was measured by cation Exchange HPLC. In breif, 10 ul of sample was injected into the HPLC. UV absorbance at 410 nm was monitored.

CBC panel
The CBC panel analysis was conducted at Biomere (Worchester, MA). Samples were processed in the IDEXX Procyte Dx analyzer.

HBG1 mRNA and Hbb-bh1 mRNA
Whole blood samples were mixed with 2x DNA/RNA shield. RNA extractions were conducted using the *Quick*-RNA Whole Blood kit from Zymo Research (# R1201). Concentrations were measured using the Qubit RNA Quantification High Sensitivity Assay (ThermoFisher Scientific # Q32855) and normalized for qPCR input. Taqman probes (ThermoFisher Scientific #4331182) for HBG1, Hs00361131_g1, and Hbb-bh1, Mm00433932_g1 were used and mRNA expression was normalized using TFRC, OAZ1 and GAPDH genes (Mm00441941_m1, Mm01611061_g1, Mm99999915_g1) respectively. TaqMan 1-Step qRT-PCR Mix (Applied Biosystems # A25602C002) was used for master mix. Expression of HBG1 and Hbb-bh1 mRNA was averaged among samples in the FTX-EEDi treatment group and compared to the average expression in the vehicle group at each time point. Percent of vehicle mRNA expression = mRNA FTX-EEDi group/ mRNA expression in the vehicle group*100.

*= p<0.05, **= p<0.01, ***= p<0.001, and ****= p<0.0001 vs vehicle control, t-test or ANOVA with Dunnett's Post-Hoc Test

CONCLUSIONS

EEDi have potential to be a transformative therapy for SCD

- Oral HbF inducers FTX-6058 and FTX-EEDi are potent and selective small molecule EED inhibitors.
- Proof of concept studies provided *in vitro* evidence for EED inhibitors in inhibiting PRC2 activity, which leads to elevation of HbF in human primary CD34+ cells.
- EED inhibitors demonstrated potent TE and HbF induction *in vivo* in animal models at plasma concentrations reasonably expected to be achieved in the clinic.
- EED inhibitors pharmacological activity in target cells can be readily monitored in the clinic since TE in bone marrow correlates with TE in peripheral monocytes in animals.
- EED inhibitors demonstrated an impressive preclinical pharmacological profile with the potential to be a disease-modifying therapeutic for patients living with sickle cell.

REFERENCES

Powars, DR et al. Is there a threshold level of fetal hemoglobin that ameliorates morbidity in sickle cell anemia? Blood. 1984 Apr; 63(4):921-6

Platt, OS et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. NEJM. 1994; 9;330(23):1639-44.

Akinsheye, I et al. Fetal hemoglobin in sickle cell anemia. Blood. 2011 Jul 7; 118(1):19-27.

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