A drug repositioning project for β-thalassemia: sirolimus and *Cinchona* alkaloids

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Introduction

The β-thalassemias are hereditary pathologies due to autosomal mutations of the β-globin gene, inducing absence or low-level synthesis of β-globin in erythroid cells and absence or low level of adult hemoglobin (HbA).

High production of fetal hemoglobin (HbF) is beneficial for β-thalassemia patients.

Drug repositioning has gained attention in the field for rare diseases, and represent a relevant novel drug development strategy.

A key advantage of drug repurposing over traditional drug development is that the repositioned drug has already passed toxicity, pharmacokinetic and pharmacodynamic tests, significantly reducing probability of project failure, time needed to reach the market and overall costs.

Figure 1

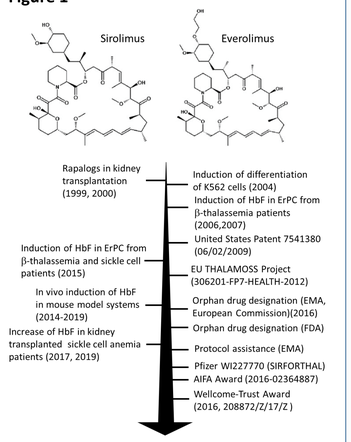
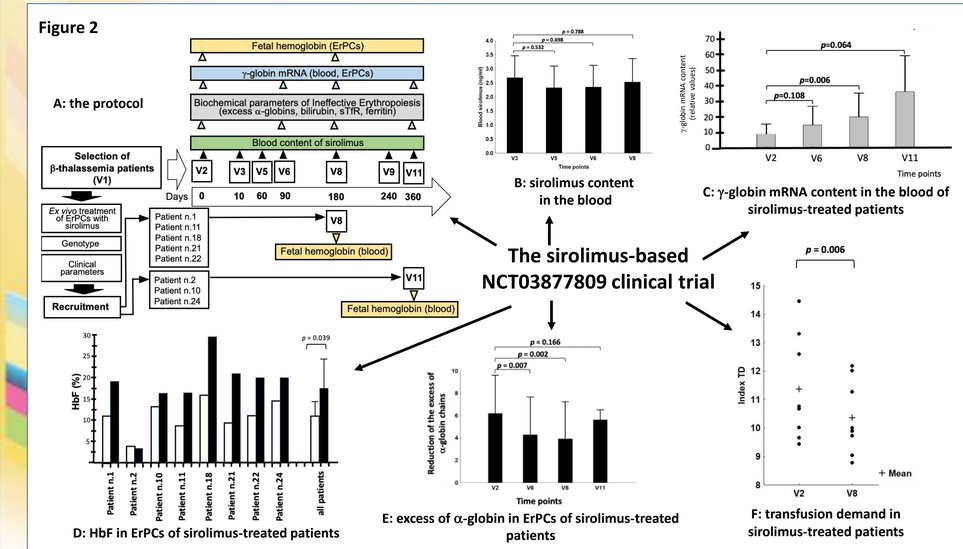


Figure 2



Expression of γ-globin genes in β-thalassemia patients treated with sirolimus: results from a pilot clinical trial (Sirthalaclin)

Sirolimus (Figure **1**), also known as rapamycin, is a lipophilic macrolide found to be a strong HbF inducer using erythroid precursor cells (ErPCs) from β-thalassemias patients as experimental model system.

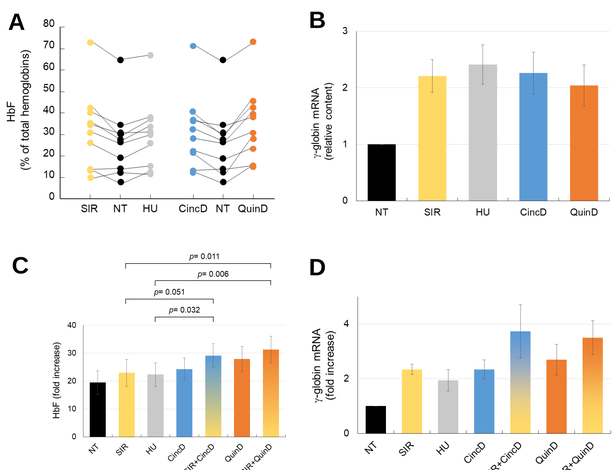
In vitro data indicate that sirolimus (and the analogue everolimus) (Fig.**1**) can be repurposed for treatment of β-thalassemia for the following reasons: (a) sirolimus increases HbF in cultures from b-thalassemia patients with different basal HbF levels; (b) sirolimus increases the overall Hb content per cell; (c) sirolimus selectively induces γ-globin mRNA accumulation, with only minor effects on β-globin and a-globin mRNAs. In vivo data confirmed the interest of sirolimus as HbF inducer in the treatment of patients with β-thalassemia.

The biochemical, molecular and clinical results of the sirolimus-based NCT03877809 clinical trial (for the protocol see Fig.**2A**) demonstrate that γ-globin mRNA content increases in the blood of β-thalassemia patients (β^+/β^+ and β^+/β^0) treated with low-dose sirolimus (1 mg/day sirolimus) (Fig.**2C**). The blood content of sirolimus is shown in Fig.**2B**). Accordingly, HbF was found increased in erythroid precursor cells (ErPCs) from treated patients (Fig.**2D**).

A second important conclusion of the trial was that sirolimus influences erythropoiesis and reduces biochemical markers associated with ineffective erythropoiesis, such as the excess of free α-globin chains (Fig.**2E**). In most of the patients a decrease of the transfusion demand index was observed (Fig.**2F**). [**1**]

Cinchona alkaloids as HbF inducers and combined treatments with sirolimus

Figure 3

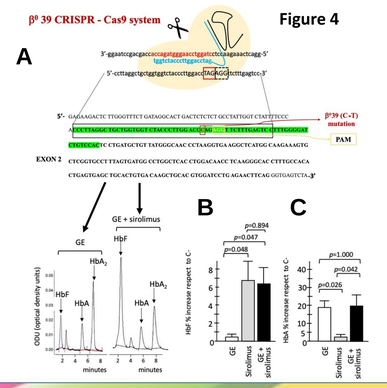


Effects of cinchonidine and quinidine on the HbF production and γ-globin gene expression in treated ErPCs. ErPCs isolated from 10 β-thalassemia patients were treated with 100 nM sirolimus (SIR), 100 μM hydroxyurea (HU), 60 μM cinchonidine (CincD) and 30 μM quinidine (QuinD) for 7 days. The lysates were analyzed by HPLC for HbF quantification (% of HbF following the different treatments with respect to total HbF produced) (Fig.**3A**) and the RNA was analyzed by RT-qPCR for determination of the relative γ-globin mRNA content (Fig.**3B**).

Cinchonidine and quinidine potentiate sirolimus-mediated induction of HbF and γ-globin mRNA in ErPCs from β-thalassemia patients

Figure **3** (**C,D**) reports the data obtained when ErPCs from 5 patients were treated with cinchonidine and quinidine in the presence of 100 nM sirolimus. The data reported are related to increase in the % of HbF (Fig.**3C**) and of γ-globin mRNA content (Fig.**3D**). The ErPC cultures exhibiting the highest levels of % of HbF and increased γ-globin mRNA are those treated with the two alkaloids and sirolimus. When the values relative to the treatments with CincD plus SIR and with QuinD plus SIR were compared to the treatment with the reference HbF inducers HU and SIR, the differences in the % of HbF increase were found to be highly significant (Fig.**3C**). [**2**]

Figure 4



Combined treatment of erythroid cells from β-thalassemia patients with CRISPR-Cas9 based genome editing of β-globin gene and the re-purposed fetal hemoglobin inducer sirolimus

Gene editing (GE) can be considered among the most promising strategy to correct hereditary mutations in a variety of monogenetic diseases, including β-thalassemia. The aim of this project was to verify whether "de novo" production of adult hemoglobin (HbA) using CRIPSR-Cas9 gene editing can be combined with HbF induction protocols based on the repurposed drug sirolimus. Gene Editing of the $\beta^0$39-thalassemia mutation was obtained by the CRIPSR-Cas9 method (Fig.**4A**); gene correction and transcription of the corrected gene has been analzyed by allele-specific droplet digital PCR and RT-PCR, respectively; production of HbA and HbF was studied by HPLC and Western blotting. The data obtained conclusively demonstrate that maximal production of HbA and HbF is obtained in GE-corrected, sirolimus-induced erythroid progenitors from $\beta^0$39-thalassemia patients (Fig.**4**, **B** and **C**).

In conclusion, GE and HbF induction might be used in combination in order to achieve "de novo" production of HbA together with increased production of induced HbF. [**3**]

References

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