Efficacy of ruxolitinib as inducer of fetal hemoglobin in primary erythroid cultures from sickle cell and beta-thalassemia patients

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Abstract

High levels of HbF may ameliorate the clinical course of β-thalassaemia and SCD. Hydroxyurea (HU) is the only HbF inducer approved for the treatment of patients. However not all patients respond to the treatment, for this reason it is noteworthy to identify new HbF inducers. Ruxolitinib is a JAK inhibitor that decreases the phosphorilation of STAT proteins. In particular STAT3 is a repressor of gamma-globin gene. The decrease of STAT3 phosphorilation could derepress gamma-globin gene and reactivate its trascription. In this study we evaluated the efficacy of ruxolitinib as inducer of HbF production. The analyses were performed in cultured erythroid progenitors from 16 beta-thalassemia intermedia (TI) and 4 sickle cell disease (SCD) patients. The use of quantitative RT-PCR technique allowed us to determine the increase of gamma-globin mRNA expression in human erythroid cultured cells treated with ruxolitinib. The results of our study demonstrated an increase *in vitro* of gamma-globin mRNA expression in almost all patients. These data suggest that ruxolitinib could be a good candidate to be used *in vivo* for the treatment of hemoglobinopathies.

Introduction

Fetal hemoglobin inhibits the polymerization of sickle hemoglobin and is a physiologic substitute for normal adult hemoglobin in erythrocytes of patients with β-thalassemia and sickle cell disease.[1-3] For this reason, in recent years, the use of fetal hemoglobin (HbF) inducing drugs became an important therapeutic strategy in reducing the clinical morbidity and mortality in patients with these diseases. It is possible to induce pharmacologically the production of HbF; several pharmacological compounds, including hydroxyurea (HU), 5-azacytidine, decitabine, sodium butyrate and rapamycin have been studied to evaluate their efficacy as HbF inducers.[4-7] Among these agents capable of stimulating fetal haemoglobin production, HU is the only drug currently used in the clinical practice of haemoglobinopathies. But not all patients respond to HU treatment, and some of responders show a decrease in their response after long-term treatment. For this reason the search of new inducers of foetal haemoglobin production is important and many new small molecule are under pre-clinical and clinical development.[8]

Primary erythroid cultures is an *ex vivo* system that recapitulates the process of erythropoiesis that *in vivo* occurs in bone marrow. Starting from the peripheral blood hematopoietic stem cells this system reproduces the differentiation of erythroid precursors to mature erythrocyte and is a good model for analyzing human erythroid cells and their functions. This system can be used for testing pharmacological modulators of foetal haemoglobin production. With this model, we developed an *ex vivo* system predictive of the *in vivo* response of patients to hydroxyurea treatment. In fact, using liquid erythroid cultures from several SCD and beta-thalassemia patients, we demonstrated that the response of the erythroid precursors exposed to hydroxyurea respect to not exposed ones, in terms of γ-globin mRNA fold increase, correlates with the HbF fold increase of the same patients treated with HU *in vivo*.[9] Ruxolitinib is a JAK inhibitor and decreases the phosphorilation of STAT (Signal transducers and activators of transcription) family proteins, in particular STAT5 and STAT3. Phosphorylation of STAT5 is essential for basal erythropoiesis and for its acceleration during stress erythropoiesis.[10] STAT3 plays an essential role in regulating gene expression of several genes involved in cell growth and apoptosis, in particular it was demonstrated to inhibit gamma-globin gene expression.[11] The decrease of STAT3 phoshorilation could derepress gamma-globin gene expression and reactivate fetal haemoglobin production. In this study we analysed the ruxolitinib ability in increasing γ-globin gene expression in primary erythroid cultures from 16 beta-thalassemia intermedia (TI) and 4 sickle cell disease (SCD) patients.

Materials and Methods

Two phase liquid primary erythroid cultures

Human erythroid liquid cultures derived from haematopoietic stem cells reproduce *in vitro* the erythropoiesis that occurs in the bone marrow. This approach allows the pharmacological treatment of erythroid-cultured precursors and to evaluate the response. After informed consent was obtained, primary cell cultures were performed using 20 mL of peripheral blood, as previously described.[12,13] Mononuclear cells from the peripheral blood were isolated by Ficoll-Hypaque (1.077 g/mL). The nucleated cells were first cultured in phase I medium, and, after six days of incubation, the nonadherent cells were resuspended in phase II medium.

Flow cytometric analysis

The phase II cultured cells were monitored for erythroid differentiation by measuring transferrin receptor (CD71) and glycophorin A antigen (GPA). The cells were washed twice in 1X PBS and stained with PE-conjugated, anti-human CD71 antibody and FITC-conjugated, anti-human GPA antibody. These analyses were performed using Cytomics FC500 Beckman Coulter.

Drug (ruxolitinib or hydroxyurea) treatment *in vitro*

At day six of phase II cells were washed with alpha-medium and the cell culture was split. Half of the culture was exposed to

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ruxolitinib or hydroxyurea. As a control, the other half of the culture was grown without the drug. For ruxolitinib a concentration of 195 nM was used, corresponding to the plasma drug concentration during *in vivo* treatment with an oral dose of 5 mg of ruxolitinib (INCB018424)[14] and for HU a concentration of 100 μM was used corresponding to the plasma drug concentration during *in vivo* treatment with an oral dose of 20 mg pro/Kg. At day ten of the culture, the cells were harvested and analysed.

Real-time quantitative PCR

RNA was isolated with TRIzol reagent, cDNA was then synthesized from total RNA using random hexamer primers. The quantitative real-time PCR assay of the transcripts was carried out in a 7900 Sequence Detector with the use of gene-specific double fluorescently-labelled TaqMan probes. To quantify globin mRNA increase between the Ct of treated samples and the Ct of the untreated samples, the comparative Ct (threshold cycle) method $2^{-(\Delta CtB-\Delta CtA)}$ was used. Before subtraction the Ct was normalized by the Ct of the endogenous reference gene, GAPDH (ΔCt).

Results

Primary erythroid cultures from 4 SCD and 16 TI patients were developed and cultured cells were exposed to ruxolitinib. To evaluate a possible effect of ruxolitinib on erythroid differentiation, cultured cells were monitored measuring transferrin receptor (CD71) and glycophorin A (GPA) antigens by flow cytometric analysis.

We found similar expression profiles in treated and untreated erythroid cells and more than 90.0% of them were positive for both markers at the end of the culture. In order to analyze the effect of ruxolitinib, at the end of the culture cells were harvested, RNA extracted and, for each sample, γ-globin mRNA fold increase with respect to untreated cells was measured by relative quantification, using a fluorescence-based q-PCR assay.

The results obtained from this analysis, summarized in Table 1, showed that ruxolitinib is able to determine a significant increase of gamma-globin gene expression, in particular a very high increase (+9 and +8) is shown in two samples (#8 and #10).

Furthermore, we wanted to compare the efficacy of ruxolitinib to HU. To this end, erythroid progenitors from 14 (4 SCD and 10 TI) of the 20 patients analyzed were exposed to HU, γ-globin mRNA fold increase was measured and the results compared to the analysis with ruxolitinib.

The results are summarized in Table 1 below and showed that ruxolitinib at 195nM is able to determine a significant increase of gamma-globin gene expression (mean value: +2.7) compared to HU (mean value: +1.8). In particular the data obtained showed that in 8 patients ruxolitinib induction was higher, in 2 patients was similar and in 4 patients was lower than HU induction.

Discussion and Conclusions

The JAK-STAT pathway mediates the effect of several growth factors and cytokines. Ruxolitinib, currently used to treat myelofibrosis, thrombocytemia and polycytemia vera is a JAK inhibitor. It was shown that treatment with ruxolitinib in patients with trasfusion dependent-tha-

Table 1. Results of the analyses.

Patient	Sex	Genotype	Gamma globin m-RNA fold increase in the presence of ruxolitinib	Gamma globin m-RNA fold increase in the presence of HU
#1	M	β039/ααα	+1	+1.5
#2	F	β039/ααα	+1.65	+1.8
#3	F	β039/β039	+1.9	+2.3
#4	F	β039/IVS1,110	+1.5	+1
#5	M	IVS1,1/ααα	+2.5	+2
#6	F	β039/IVS1,6	+1.7	
#7	M	IVS1,6/frcd9	+3	
#8	F	β039/IVS1,6	+9	+1
#9	M	IVS1,1/β039	+2.2	
#10	M	δβ/IVS1,110	+8	
#11	F	δβ/IVS1,110	+1.8	
#12	F	IVS2,1/ααα	+3.9	+3
#13	M	β039/-101	+1.4	+1.5
#14	M	IVS1,6/β039	+1	
#15	M	IVS1,6/IVS1,6	+2	+1.3
#16	F	cod39/IVS2,1	+4.2	+1
Sickle cell anemia				
#17	F	βS/β039	+1.6	+2.1
#18	M	IVS1,6/βs	+2.5	+1
#19	M	β039/βs	+6	+4
#20	M	βs/IVS1,110	+1.5	+1.8

lassemia led to a sustained reduction in spleen size, and, hence, could be considered as an option for trasfusion dependent-thalassemia patients with splenomegaly.[15]

It decreases the phosphorilation of STAT family proteins, in particular STAT3[14] and STAT5.[10] During erythropoiesis, STAT5 is involved in the signal transduction mediated by erythropoietin (EPO). STAT3 has been implicated in gamma-globin gene regulation.

In particular it was shown that STAT3 serves as a physiologically relevant repressor of gamma-globin gene expression.[6] The decrease of STAT3 phosphorilation could decrease the inhibition of gamma-globin gene expression leading to the reactivation of fetal hemoglobin production.

In this study we evaluated *in vitro* the efficacy of ruxolitinib as a Hb F inducer in sickle cell anemia and β-thalassemia intermedia patients. To this end, we analyzed ruxolitinib effect in cultured erythroid precursors from 4 sickle cell disease and 16 TI patients. We found that ruxolitinib was able to increase γ-globin mRNA expression more than HU, the only drug used in clinical practice of hemoglobinopathies to induce HbF production. We are planning to perform studies on the analysis of HbF increase. The *in vitro* used ruxolitinib concentration of 195nM corresponds to the plasma drug concentration during the *in vivo* treatment with an oral dose of 5 mg of ruxolitinib (INCB018424).[11] For this reason it could be in principle usable *in vivo* for the treatment of haemoglobinopathies, particularly in patients who do not respond to HU therapy or who show a decreased response after long-term treatment.

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