Cultures of erythroid precursors in the screening of gamma-globin mRNA and fetal haemoglobin inducers used for a new approach in thalassemia and sickle cell anemia therapy

Nicoletta Bianchi[2], Cristina Zuccato[2], Carlo Mischiati[2], Ilaria Lampronti[2], Monica Borgatti[2], Eugenia Pruss[1], Eitan Fibach[1] and Roberto Gambari[2,3]

[1]Department of Haematology, Hadassah, Hebrew University Medical Centre, Jerusalem, Israel;
[2]Gentech-For-Thal, Department of Biochemistry and Molecular Biology, University of Ferrara, Via Fossato di Mortara 74, 44100 Ferrara, Italy
[3]Laboratory for the Development of Pharmacological and Pharmacogenomic Therapy of Thalassaemia,Biotechnology Centre, Via Fossato di Mortara, 64/b, 44100 Ferrara, Italy

INTRODUCTION

Several investigations have demonstrated since a long time that the clinical status of patients with beta-globin disorders can improve by pharmacologically increased fetal gamma-globin genes expression. In addition, levels greater than 9% could reduce early mortality (1). A large number of compounds have been considered for stimulation of HbF (fetal hemoglobin), such as hormones, hematopoietic growth factors and cytokines (2), chemotherapeutic agents including 5-azacytidine and hydroxyurea (HU) (3,4). However, cytotoxicity, potential carcinogenicity and the moderate effects obtained have limited their clinical use. Our group studied several inducers of erythroid differentiation (5, 6). In the present study we demonstrate that rapamycin induces an increase of HbF production in erythroid precursors (7). This compound is able to increase gamma-globin gene expression in stem cells from blood of normal subjects (7) as well as beta-thalassemia patients (8). The proportion of increased HbF by pharmacological treatment was determined by HPLC and globin mRNAs were quantify by RT-PCR analysis. In this context, *in vitro* cultures of erythroid precursors represent a model to study HbF induction under physiological conditions (9). This approach can be used for the screening of potential erythro-differentianting drugs, for useful applications in therapy of hematological disorders including beta-thalassemia and sickle cell anemia.

Fig. 1. This representation of world distribution of thalassemia is changed in the last century further to migration of people.

The Rapamycin was isolated in the 1975 from samples of yeast (*Streptomyces igroscopicus*) found on Rapa Nui island.
The Rapamycin is used for its antiproliferative effects:

- Immunosuppressant (in the transplants, after angioplasty and in rheumatoid arthritis).
- Chemiotherapic agent (in the treatment of mieloma multiple, tumor of the colon and rectum, breast, kidneys, pancreatic and methastatic cancers)
- Antiviral agent (repressor of HIV-1 replication).

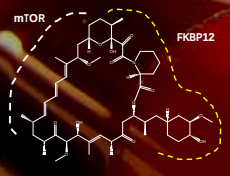


Fig. 2. Chemical structure of Rapamycin. The molecule is a lipophilic macrolide lactone, also called sirolimus. Rapamycin is a complicated molecule comprising a 31-membered ring including a pipecolinyl group and pyranose ring, a conjugated triene system and a tri-carbonyl region. Also, it has 15 chiral centres, meaning the number of possible stereoisomers is enormous.

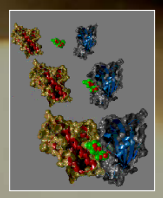


Fig. 3. Interactions of Rapamycin with targets mTOR and FKBP12.

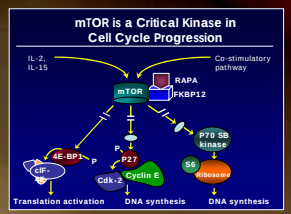
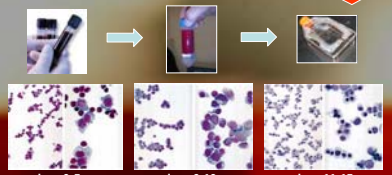


Fig. 4. Reported effects for Rapamycin.

Rapamycin (green, in Fig. 3) penetrates the cell membrane and binds to an intracellular receptor called FKBP (FK506 Binding Protein, the blue protein). Then this complex binds to FRAP (FKBP Rapamycin Associated Protein, the red protein) inhibiting mTOR functions, also the phosphorilation of its targets: the proteins p70S6K and 4E-BP1 (Fig. 4). This process blocks the cell cycle in the G1 phase and increases the expression of p27, an inhibitor of cyclin-dependent kinase, and p21, a related protein to NF-E2 and GATA-1 genes involved in erythroid differentiation.

MATERIALS AND METHODS

Fig. 5. Two-phase Liquid culture system of erythroid precursor cells. Peripheral blood samples were obtained from patients with beta-thalassemia. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and seeded in alfa-MEM supplemented with 10% FBS, 10% conditioned medium from the 5637 bladder carcinoma cell line and 1 mg/ml of cyclosporine A (phase I). In the phase II, the non adherent cells were harvested in fresh medium with 30% FBS, 1% deionized BSA, 10^{-5} M beta-mercaptoethanol, 2 mM glutamine, 10^{-6} M dexamethasone, 1 U/ml of Erythropoietin (Epo) and 10 ng/ml of stem cell factor. The cultures were incubated at 37°C, under an atmosphere of 5% CO_2 with extra humidity (9).



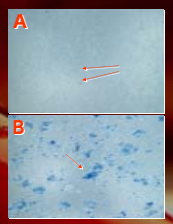


Fig. 6. Staining of erythroid precursor cells by benzidine/H_2O_2. In the figure are arrowed the benzidine/H_2O_2 positive cells, which sinthesize hemoglobins: in A, is reported an example of untreated control culture and in B, the same culture treated with an erythroid differentiation agent.

Fig. 7. Description of quantitative real time PCR reaction.

Fig. 8. Quantification of HbF production by HPLC analysis.

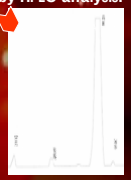


Table 1. Sequence of primers and fluorescent-labelled probes used in the real-time quantitative PCR assays.

primer forward alfa-globin	5'-CACGCGCACAAGCTTCG-3'
primer reverse alfa-globin	5'-AGGGTCACCAGCAGGCAGT-3'
probe alfa-globin	5'-FAM-TGGACCCGGTCAACTTCAAGCTCCT-TAMRA-3'
primer forward beta-globin	5'-CAAGAAAGTGCTCGGTGCCT-3'
primer reverse beta-globin	5'-GCAAAGGTGCCCTTGAGGT -3'
probe beta-globin	5'-FAM-TAGTGATGGCCTGGCTCACCTGGA-TAMRA-3'
primer forward gamma-globin	5'-TGGCAAGAAGGTGCTGACTTC-3'
primer reverse gamma-globin	5'-TCACTCAGCTGGGCAAAGC-3'
probe gamma-globin	5'-FAM-TGGGAGATGCCATAAAGCACCTGC-TAMRA-3'

RESULTS

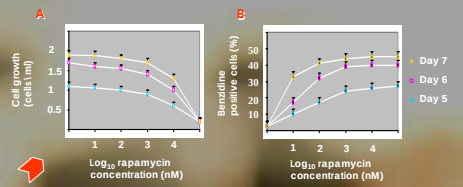


Fig. 9. The relationship between the concentration of rapamycin and effects on cell growth (A) and erythroid differentiation (B) in K562 cells. The induction of erythroid differentiation increases with increasing of the rapamycin doses and the concentrations used were unable to inhibit cells proliferation.

Table 2. Characterization of beta-thalassemia patients used for cultures of erythroid precursors treated with 100 nM Rapamycin.

patient number	thalassemia type	mutation	blood transfusion	splenectomy
1.	β^+/β^+	IVS1-110/IVS1-110	+	-
2.	β^0/β^0	IVS2-1/IVS2-1	+	+
3.	β^+/β^0	-28/FS44	+	-
4.	β^+/β^0	FS36-37/FS44	+	-
5.	β^+/β^+	IVS1-6/IVS1-6	+	+

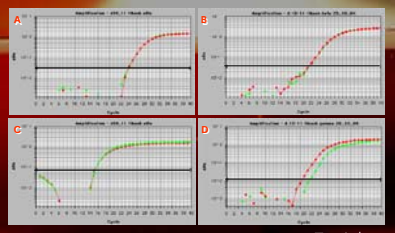


Fig. 10. Quantitative RT-PCR analysis of globin transcripts in Rapamycin-treated erythroid precursors from thalassemic patients. In A, were reported the kinetics of GAPDH PCR-products generation; in B, beta-globin products; in C, alfa-globin products and in D, gamma-globin products. In red was indicated the cells treated with 100 nM Rapamycin and in green the untreated cells.

Table 3. Induction of globin mRNAs accumulation in Rapamycin-treated erythroid precursors with respect to untreated cells.

patient number	induction mRNA γ	induction mRNA β	induction mRNA α	mRNA γ/β
1.	1,37	0,92	1,18	1,49
2.	1,32	1	1,04	1,32
3.	2,53	0,83	1,49	3,05
4.	4,72	1,02	0,95	4,63
5.	1,01	0,47	0,77	2,15

patient number	HbF (%)		Total Hb (pg/cell)	
	control	+Rapamycin	control	+Rapamycin
1.	20,9	26,0	5,9	7,4
2.	93,4	97,7	8,3	9,9
3.	15,4	28,9	5,0	3,1
4.	34,5	43,1	7,2	7,3
5.	44,6	59,0	4,7	10,8

Table 4 and Table 5. Quantification of fetal hemoglobin by HPLC analysis. The effects were valued on untreated and treated with Rapamycin cells.

patient number	HbF (pg/cell)	
	control	+Rapamycin
1.	1,23	1,92
2.	7,75	9,67
3.	0,77	0,89
4.	2,48	3,15
5.	2,10	6,37

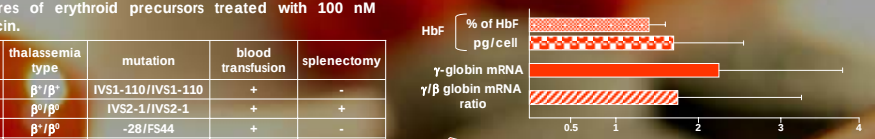


Fig. 11. Accumulation of specific gamma-globin mRNA and fetal hemoglobin. Increase of HbF (% of total Hb and pg/cell), gamma-globin mRNA content and gamma-globin/beta-globin mRNA ratios following treatment with Rapamycin of erythroid precursor cells from beta-thalassemia patients. The data were expressed as fold increase with respect to untreated cultures and were average ±SD of 5 considered samples.

CONCLUSIONS

- Rapamycin increases preferably gamma-globin RNA accumulation in treated erythroid progenitors from beta-thalassemia patients.
- Rapamycin induces HbF production (when the data are considered as %Hb or pg/cell) in this cellular culture system.
- Also, Rapamycin represents potential therapeutic drug in the therapy of thalassemia diseases.

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