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Innovative strategies for a personalized therapy of β-thalassemia and sickle cell anemia

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ABBREVIATIONS

5-MOP	5-methoxypsolaren
ANG	Angelicin
B+	Blue positive cells to benzidine/H_2O_2
BCL11A	B-cell lymphoma/leukemia 11A
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CBT	Cord blood transplantation
CHD	Coronary heart diseases
CML	Chronic myelogenous leukemia
DFO	Desferrioxamine
DFP	Deferiprone
DFT	Desferrithiocin
DMSO	Dimethylsulfoxide
EKLF	Erythroid Krüppel-like factor
FKLF	Krüppel-like zinc finger protein
FOG1	Friend of GATA Protein 1
GAPDH	Glyceraldehyde phosphate dehydrogenase
GATA-1	Erythroid cell- and megakaryocyte-specific transcription factor 1
GC-MS	Gas chromatography-Mass spectrometry
GSH	Reduced glutathione
HCS	Hematopoietic stem cell
HMBA	Hexamethylene bisacetamide
HPFH	Hereditary persistence of fetal hemoglobin
HPLC	High performance liquid chromatography
HU	Hydroxyurea
MEL	Murine erythroleukemia cell
MTH	Mithramycin
NF-E2	Nuclear factor erythroid 2
Oct-1	Octamer transcription factor
PDI	Polydispersity index
PNA	Peptide nucleic acid
PUVA	Psoralens + Ultraviolet A radiation

QTR	Quantitative Trait Loci
RBC	Red blood cell
ROS	Reactive oxygen species
RPL13A	Ribosomal protein L13A
RSV	Resveratrol
Sp1	Transcription factor specificity protein 1
SCA	Sickle-cells anemia
SCF	Stem cell factor
SCL	Stem cell leukemia gene
TF	Transcription factor
TMA	4,6,4' –trimethylangelicin
USF	Upstream stimulatory factor
UV	Ultraviolet
VOE	Vaso-occlusive episodes
α-MEM	Minimum essential medium with alpha modification
β-ME	β-mercaptoethanol

INTRODUCTION

1. HEMOGLOBIN

1.1. Structure and functional characteristics

Mammalia hemoglobin are tetrameric molecules of 64000 daltons with a diameter of approximatively 5.5 nm, composed of two identical pairs of polypeptide chain, the globin subunits. Each chain has a molecular weight of about 16000 daltons and contains one heme prosthetic group, attached via a histidine side chain. (Surgenor, 1975; Steinberg et al., 2009). The most common form of adult hemoglobin, HbA consists of two alpha and two beta subunits (α_2 β_2) (Figure 1A). However a second similar but less abundant form is also found, HbA_2, with two delta subunits replacing the beta ($\alpha_2\delta_2$) The three-dimensional structure of hemoglobin was solved by Max F. Perutz in 1960 (Perutz, 1960). The major functions of Hb are to transport oxygen (O_2) from the lungs to peripheral tissues and carbon dioxide (CO_2) from the tissues to the lungs. The kinetics of Hb-O_2 binding and release are fine-tuned for this purpose and adaptable according to developmental ontogeny and metabolic perturbations (Thom et al., 2013).

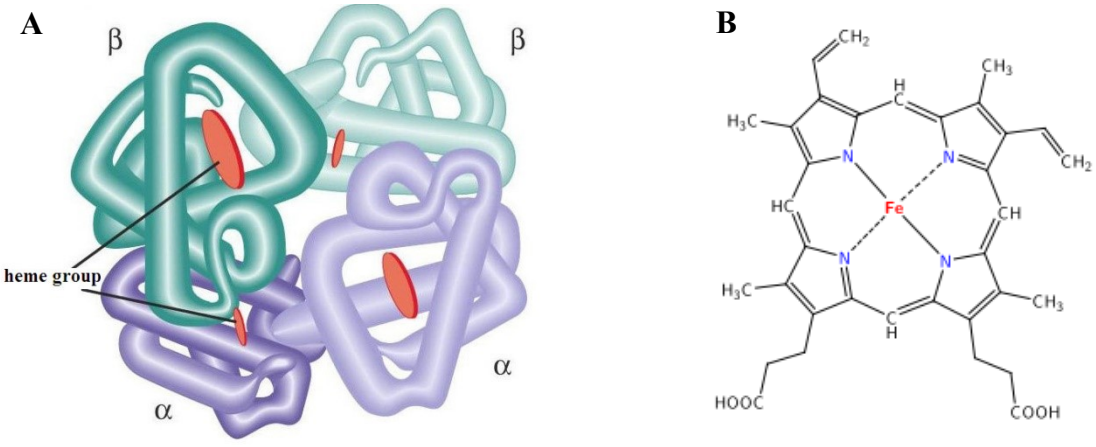


Figure 1. Structure of hemoglobin (A) and heme prosthetic group (B). Hemoglobin is a heterotetramer composed of α-like and β-like globin subunits, each bound to a heme prosthetic group. The prosthetic group is the iron complex of protoporphyrin IX.

The prosthetic group heme (Figure 1B) is a complex of iron with protoporphyrin IX, that is formed by four pyrrole rings connected by methine bridges and with the external

position substituted by methyl, vinyl and propionic acid groups. The iron atom occupies the central position of the porphyrin ring and is coordinated to the four pyrrole nitrogens. Iron must be in its reduced (ferrous, Fe^{2+}) state for Hb to bind O_2. Oxidized or "met" Hb (ferric, Fe^{3+}) cannot bind O_2 and is relatively unstable, tending to lose hemin and denature. (Surgenor, 1975; Steinberg et al., 2009).

Within the hemoglobin, each globin chain binds the unlike subunit through two distinct interfaces, termed α1β1 and α1β2. Individual globin chain form dimers through the extremely high affinity α1β1 interaction. The α1β2 interaction, which is lower affinity, mediates tetramerization. Globin chain monomers tend to form intracellular precipitate because they are relative unstable. Mutations that impair the α1β1 interaction can cause erythrotoxicity and as a consequence hemolytic anemia by favoring the accumulation of monomeric subunits. Oxygen binding, which destabilizes the α1β2 interaction, results in a transition of the quaternary structure from the "T" (tense, low affinity, deoxygenated) to "R" (relaxed, affinity, oxygenated) state. This process is the basis for oxygen cooperativity, a phenomenon discovered by Christian Bohr who claimed that the affinity of a hemoglobin tetramer for O_2 increases as more O_2 is bound (Bohr et al., 1904). Hemoglobins accumulate in extraordinarily high concentrations within developing erythroblasts and mature erythrocytes. The intact hemoglobin tetramer is sufficiently soluble to remain in the fluid phase under these conditions.

1.2. The globin genes

In humans, two gene clusters direct the synthesis of hemoglobins: the α locus, which contains the embryonic ζ gene and the two adult α genes α1 and α2, which produce identical globin, but differ in the amount of mRNA transcript that is greater for α2; and the β locus in which reside five beta-like globin genes: ε-globin, expressed during early embryogenesis in primitive erythrocytes derived from the yolk sac; ^{A}γ- and ^{G}γ-globin, expressed from mid-gestation until infancy; and δ- and β-globin which compose the minor and major forms of adult hemoglobin, predominating after birth (Figure 2). The switches from ε to γ and from γ to β globin gene expression are controlled exclusively at the transcriptional level (Stamatoyannopoulos et al., 2001), while the ζ to α switch is also controlled at posttranscriptional level (Liebhaber et al., 1996).

The cluster α is about 30 Kb and it is located in the distal portion of the short arm of chromosome 16, at the level of the band 16p13.3. The region occupied by the cluster α is a variable and unstable region, rich in GC (guanine-cytosine) in a percentage of 54%, with an open chromatin configuration and a high density of adjacent non-globin genes constitutively expressed.

The cluster β is about 70 Kb and it is located in the distal region of chromosome 11, corresponding to the band 11p15.5. The human β-globin locus contains a powerful set of enhancer elements, termed the β locus control region (βLCR), upstream (5′) of the β locus structural genes (Tuan et al., 1985). LCR is a region of approximately 25 kb located 6 to 18 kb upstream of the gene for the ε-globin, which is essential for gene expression and influent in the structure of chromatin. The LCR has five sites hypersensitive to DNase I (5'HS), which makes this region accessible for the transcription and for chromatin remodeling factors. HS sites have binding sites for specific transcription factors such as GATA-1 (erythroid cell- and megakaryocyte-specific transcription factor 1), essential for the erythroid developing (Grosveld et al., 1993), Sp1 (transcription factor specificity protein 1), NF-E2 (nuclear factor erythroid 2) and USF (upstream stimulatory factor). LCR functions may affect the basic transcription machinery directly. RNA polymerase II (pol II), one of the essential components of the eukaryotic transcription apparatus, was found to be associated with the β-globin LCR in a p45/NF-E2-independent manner, whereas its recruitment to the promoter required p45/NF-E2. These data suggest that pol II accesses the LCR and p45/NF-E2 induces long-range transfer of pol II to the promoter, resulting in transcriptional activation (Johnson et al., 2001).

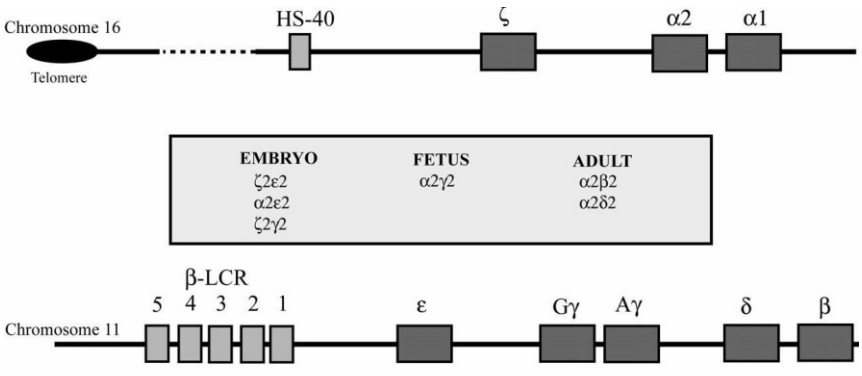


Figure 2. The human α- and β-globin locus. ζ, α2, and α1 globin genes situated near the telomeric region of the short arm of chromosome 16. About 40 kb upstream of the 5′ end of the ζ globin gene is the HS-40 α-globin genes remote regulatory region. The β-like globin cluster is located on the short arm of chromosome 11; upstream of the cluster is the β-globin gene remote regulatory locus control region (β-LCR). In between are represented the different ontogenetic stages of Hb.

1.3. Developmental modulation of globin genes

Individual globin genes are expressed at different levels in developing erythroblasts of human embryos, fetuses and adults (Figure 3). During the embryonic period, three types of hemoglobin are synthesized in the yolk sac: Hb Gower1 ($\zeta_2\varepsilon_2$), Gower2 ($\alpha_2\varepsilon_2$) and Hb Portland ($\zeta_2\gamma_2$), which have higher affinity for oxygen than fetal hemoglobin and adult hemoglobin. The expression of the ζ-globin genes decreases after the first two weeks of gestation, while the expression of α-globin genes increases. The replace of ε-globin with γ-globin happens after about six weeks of gestation. Between the 8th and 10th week of gestation, erythropoiesis take place in the liver, the gradual inactivation of ζ and ε genes occurs and and the α and γ chains start to be produced (first switch). During the fetal period, HbF ($\alpha_2{}^G\gamma_2$ and $\alpha_2{}^A\gamma_2$) represents 90% of total hemoglobin produced in this stage. Its production continues even after birth, constituting 5% of the total hemoglobin for the first months of life. The synthesis of γ-globin is gradually reduced and, at the same time, the synthesis of β- globin chains starts. Around the 8th-10th week of gestation, β-globin production increases (second switch) as well as the production of δ-globin. Hence, the adult produces two different types of adult hemoglobin: 98% is HbA ($\alpha_2\beta_2$) and the remaining 2% is HbA$_2$ ($\alpha_2\delta_2$). The fetal hemoglobin (HbF) is present only in traces in adults, but its levels can vary significantly depending on several factors such as sex, age, or point mutations in the β-cluster or genes related to it.

Table 1. Different types of human hemoglobin during the development.

Hemoglobin Type	Structure	Developmental Stage
Hb Gower 1	$\zeta_2\ \varepsilon_2$	embryo
Hb Gower 2	$\alpha_2\ \varepsilon_2$	embryo
Hb Portland	$\zeta_2\ \gamma_2$	embryo
HbF	$\alpha_2\ \gamma_2$	fetal and adult
HbA2	$\alpha_2\ \delta_2$	adult
HbA	$\alpha_2\ \beta_2$	adult

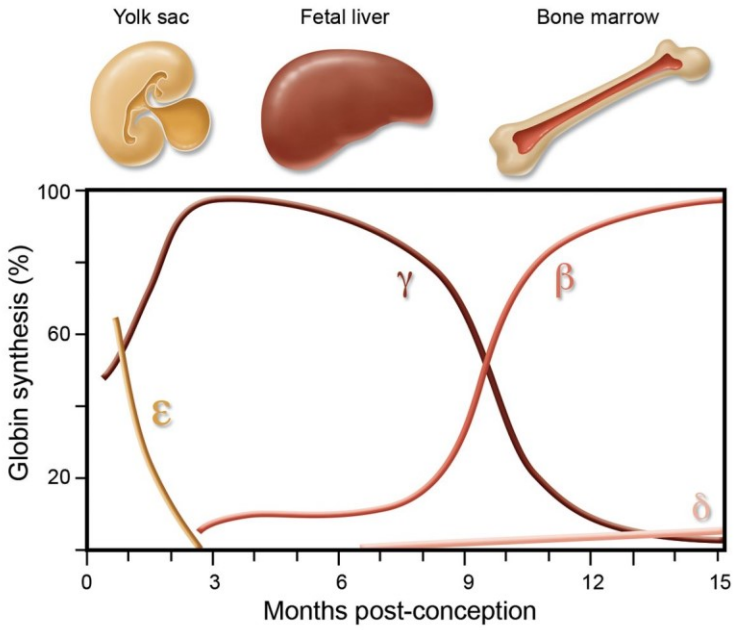


Figure 3. β-like globin protein content during development in human.
The globin producing tissues at the stage of development are indicated above the graph.

Discovery of the LCR had a major impact on the investigation of mechanisms of switching. It has been demonstrated that during the fetal stage of development, there is a preferential interaction between the γ genes and the LCR; the β globin genes are turned off competitively. On the other hand, the LCR interacts preferentially with the β gene during the adult stage of development, resulting in silencing of the γ globin genes (Enver et al., 1990). Sequences participating in the silencing process were identified in both the proximal and the distal ε gene promoter: GATA1 and YY1 have been described as developmental repressors of the human ε-globin gene (Raich et al., 1995). The proximal ε gene promoter (as well as the γ promoter but not the other globin gene promoters) contain direct repeat sequences (DR1 box) located near the CAAT box. These DR elements bind a factor, which is identical to the orphan nuclear receptor COUP-TF and participate in ε gene silencing (Filipe et al., 1999).

A mutation in a GATA site in the−378 to −730 sequence was found to produce the phenotype of hereditary persistence of fetal hemoglobin, further suggesting that this upstream region contributes to γ gene silencing *in vivo* (Luo et al., 2004). EKLF (erythroid Krüppel-like factor) binds the CACCC sequence of the β-globin gene with high affinity and it is considered a determining factor for switching (Asano et al., 1998).

2. β-THALASSEMIA

2.1. Definition

Beta-thalassemia syndromes are a group of hereditary autosomal recessive blood disorders characterized by anomalies in the synthesis of the β chains of hemoglobin resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals. The first clinical description of thalassemia appeared in 1927 and it is attributed to the pediatricians Thomas B. Cooley and Pearl Lee (Cooley et al., 1927). The term thalassemia is derived from the Greek, *thalassa* (sea) and *haima* (blood).

β-thalassemia is distributed mainly in Mediterranean countries, the Middle East, Central Asia, India, Southern China, and in countries along the north coast of Africa and in South America. Population migration and intermarriage between different ethnic groups have introduce thalassemia in almost every country of the world, including Northern Europe. The highest frequency of β-thalassemia is reported in Cyprus (14%), Sardinia (10,3%) and Southeast Asia. About 1.5% of the global population (80 to 90 million people) are carriers of β-thalassemia, with about 60,000 symptomatic individuals born annually, the great majority in the developing world. The total annual incidence of symptomatic individuals is estimated at 1 in 100,000 throughout the world and 1 in 10,000 people in the European Union (Galanello et al., 2010).

According to the hematological and severity of symptomatic events, thalassemias are distinguished in three main forms: *Thalassemia Minor* also called "Beta-thalassemia carrier", "Beta-thalassemia trait" or "Heterozygous beta-thalassemia", *Thalassemia Intermedia* and *Thalassemia Major*, variably referred to "Cooley's Anemia" and "Mediterranean Anemia".

Carriers of *thalassemia minor* have usually no relevant symptoms but sometimes have a mild anemia. This individuals have a greater number of red blood cells than the normal subjects, but RBCs are a little bit smaller (hence the term microcythemia) and poorest of hemoglobin (around 15% less than the normal RBCs). Moreover, HbA_2 level is increased (Weatherall, 1986; Cao et al., 2010).

Patients with *thalassemia major* have a severe microcytic and hypochromic anemia, associated with increased number of red blood cells and low mean corpuscular volume (MCV). Clinical presentation of thalassemia major occurs between 6 and 24 months. The clinical picture of thalassemia major is characterized by growth retardation, pale skin, poor

musculature, hepatosplenomegaly, leg ulcers, skeletal changes resulting from expansion of bone marrow. If a regular transfusion's program that maintains a minimum Hb concentration of 95–105 g/L is initiated, then growth and development are normal until the age of 10–11 years. After the age of 10–11 years, affected individuals are at risk of developing severe complications related to post-transfusional iron overload, depending on their compliance with chelation therapy.

Individuals with *β-thalassemia intermedia* show a markedly heterogeneous clinical picture. Principle symptoms are pallor, liver and spleen enlargement, moderate to severe skeletal changes, hyperplastic erythroid marrow, a tendency to develop osteopenia and osteoporosis, and thrombotic complications. Patients have a milder anemia and do not required or only occasionally require transfusion (Galanello et al., 2010; Cao et al., 2010).

A well-recognized mechanism for the production of a milder phenotype is the inheritance of a mild or silent β-thalassemia mutation, in homozygosity or in a compound heterozygous state with a severe mutation. Mild and silent mutations are molecular defects of the β-globin gene associated with a consistent residual output of β-globin chains from the affected β-locus. A second characterized molecular mechanism is the co-inheritance with homozygous β-thalassemia of mutations of the α-globin gene associated with α-thalassemia which by reducing the output of α-chains decrease the α/nonα chain imbalance. Finally, HPFH determinants were recently shown to play a major role in determining the phenotype of β-thalassemia (Cao et al., 2011).

2.2. Genetic alteration

The alterations that can affect the human β-globin gene responsible for thalassemia are represented by deletions, defects in gene transcription, defects in pre-mRNA maturation (splicing alterations, mutations in the cleavage site and polyadenylation of pre-mRNA), defects in the translation of mRNA into protein (start codon mutations, nonsense mutations, frameshift mutations in the 3' UTR region) and defects that compromise the stability of the β-globin chain.

Mutation in the promoter region: these mutations reduce the amount of β-chain (β+-thalassemia) and involve the TATA box regions at the level of nucleotides -28, -29, -30, -31, the CACCC box proximal at the level of nucleotides -86, -87, -88, -89, -90, -92 and CACCC box distal at the position -101.

Splicing process alteration: the mutation that affect the invariant dinucleotides GT or AG at the exon–intron splice junction completely abolish normal splicing and produce the phenotype of β^0-thalassemia. These mutations can be base substitutions that change one or the other of invariant dinucleotides or short deletions that remove them. Mutations within the consensus sequences at the splice junctions reduce the efficiency of normal splicing to varying degrees and produce a β-thalassemia phenotype that ranges from mild to severe. For example, mutations at position 5 IVS1 G → C, Tor A, considerably reduce splicing at the mutated donor site compared with normal. The mutations appear to activate the use of three "cryptic" donor sites, two in exon 1 and one in IVS1, which are used preferentially to the mutated donor site (Treisman et al., 1983).

Mutations causing abnormal posttranscriptional modification: Proper cleavage of the primary RNA transcript and polyadenylation of the 30 end of the mRNA is guided by a consensus hexanucleotide sequence (AATAAA) about 20 nucleotides upstream of the poly-A tail. Mutations affecting the AATAAA sequence include seven base substitutions at different locations; two short deletions of 2 and 5 bp each, and one deletion of the total AATAAA sequence. These mutations markedly decrease the efficiency of the cleavage-polyadenylation process, only about 10% of the mRNA is properly modified (Orkin et al., 1985). Therefore, the associated phenotype is that of β^+-thalassemia of moderate severity. Mutations affecting other sites in the 30 UTR, a C→G substitution at nucleotide 6, and a 13 bp deletion at nucleotides 90 downstream from the termination codon, also result in β^+-thalassemia (Rund et al. 1992; Thein, 2013).

Defects in RNA translation: Nonsense mutations, or frameshift mutations within the start codon, in exons and termination codon or in the 3' UTR region, can cause more or less early arrest of the translation of messenger in protein. Mutations in the start codon cause severe β^0-thalassemias, while mutations involving the stop codon or region 3' UTR generate β^+-thalassemias. The nonsense mutations consist of the replacement of a purine base in a codon of the coding DNA and gives rise to the formation of a termination codon (TAA, TAG or TGA), and hence the arrest of the RNA translation. β-globin chains are not formed at all and the phenotype is that of a very significant β^0-microcythemia. The frameshift mutations consist of deletions or insertions of one or a few nucleotides in the DNA chain, causing the shift of the reading frame, with more or less early arrest of translation. β-globin chains synthesized appear totally altered, inducing the expression of a β^0-thalassemic phenotype. These types of mutations mainly affect exons, but may also

involve the start codon, the stop codon and the 3' UTR region, generating in the last two cases a β^+-thalassemia.

Deletion of the β-globin gene: this mutations are very rare, except for a 619-bp deletion removing the 3′ end of the beta globin gene, which is relatively common in India and Pakistan. Another group of deletions (complex beta-thalassemia), in addition to the beta globin gene, involve also the delta (δ-β^0-thalassemia), the δ and $^A\gamma$-γ genes ($^G\gamma$-$^A\gamma\delta\beta^0$-thalassemia), or the whole beta globin gene cluster. Finally, partial or total deletions of the LCR, but leaving the β-globin gene intact, inactivate the β-globin gene (Cao et al., 2011).

The most common alterations in Italy are CD39 C→T (β^0 39), β^+ IVS-I-110 G→A, β^+ IVS-I-6 T→C, β^+ IVS-I-1 G→A and β^0 IVS-II-1 G→A.

- β^0 39 C→T. It consists of a nonsense point mutation that converts the 39 CAG codon of the β-globin gene coding for glutamine, in a TAG codon, which imparts the termination signal to the globin synthesis. In conditions of homozygosis, this alteration causes thalassemia major.
- β^+ IVS-I-110 G→A. This mutation is caused by the substitution of a guanine with adenine in the consensus sequence. The sequence is located in the first intron of the β-globin gene, 19 nucleotides away from the splice site AG. As a result of the substitution a cryptic splicing site is activated causing a defect in the recognition of the region of exon/intron junction (Orkin et al., 1982). The mutation causes severe β^+-thalassemia, but in conditions of homozygosis or heterozygosis for other defects involving the β-globin gene, both β-thalassemia major and β-thalassemia intermedia may occur.
- β^+ IVS-I-6 T→C. It involves the consensus sequence of the splicing donor site of the first intron of the β-globin gene, where a thymine, localized nearby the CT dinucleotide in the 5' intron, is replaced with a cytosine. This results in the alteration of the conserved sequence and a lower efficiency of the processing of pre-mRNA (Husman, 1993).
- β^+ IVS-I-1 G→A. A guanine of the invariant GT dinucleotide in 5' of the first intron of the β-globin gene is exchanged with adenine, causing the destruction of the normal splice site, abolition of processing and cancellation of normal mRNA production. The phenotype that results is the β^0-thalassemia with a clinical situation rather serious that may involve β-thalassemia major both in homozygous than in heterozygous (Le Denmat, 1997).

- β^0 IVS-II-1 G→A. It is caused by a point mutation in position 1 of the second intron of the β-globin gene and causes β^0-thalassemia.

2.3. Pathophysiology and clinical complications

The β-thalassemias are characterized by a quantitative reduction or total absence of the β-globin chain synthesis: the result of this event is respectively the decrease and the lack of adult hemoglobin (HbA, $\alpha_2\beta_2$). Unbalanced α and β globin production has an even more profound impact upon clinical severity. Unpaired insoluble a globin chains accumulate and precipitate, forming inclusion bodies. The α-globin monomers are degraded via several pathways triggered by ROS to result in the release of hemin (heme containing oxidized ferric iron) and free iron, both able to participate in redox reaction (Shaeffer, 1988). Further ROS are generated damaging cellular protein, lipids and nucleic acids (Schrier, 1997). Ineffective erythropoiesis and death of bone marrow erythroid precursors appear to be a result of enhanced activation of the apoptosis pathways (Ribeil et al., 2013), including premature degradation of GATA1 (Mettananda et al., 2015) (Figure 4).

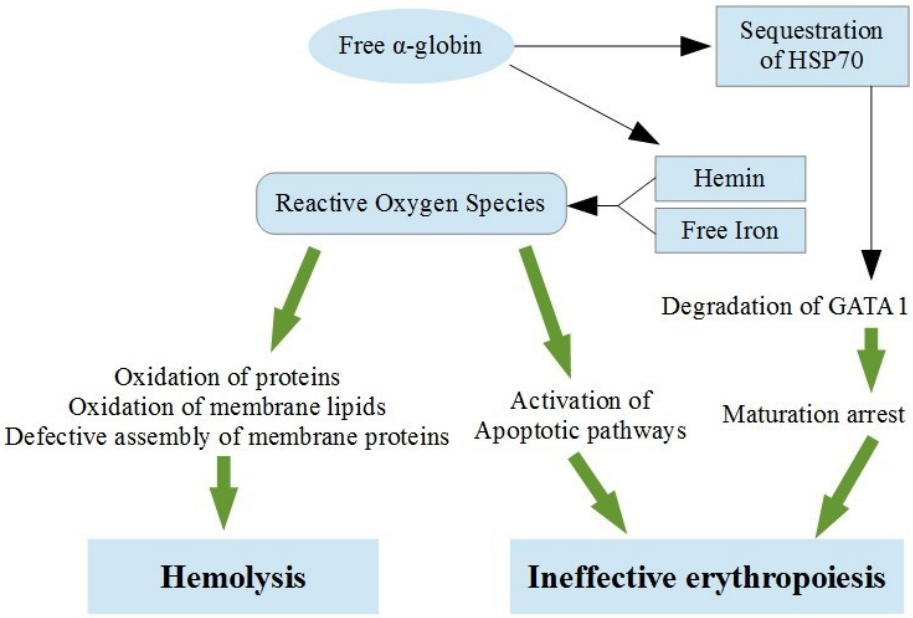


Figure 4. Pathophysiological mechanisms due to the accumulation of α-globin chain in β-thalassemia. The generation of ROS, triggered by the unbalanced excess of α-globin results in hemolysis of mature red blood cells and ineffective erythropoiesis

The small numbers of erythroblasts that survive maturation carry the burden of α-globin inclusions into the circulation. These damaged erythrocytes are prematurely destroyed by the RE cells of the spleen and liver, producing hemolytic anemia. Massive erythroid hyperplasia occurs as a compensatory response to the severe anemia, but very few mature red cells are produced. The compensatory response is thwarted, however, by the premature death of erythroid progenitors. Masses of hyperactive erythroid marrow invade bony cortices, causing pathologic fractures, marked facial and other bony deformities. Extramedullary hematopoiesis occurs in the pelvic basin and thoracic cavity (Benz, 1996). Patients with chronic anemia increase their cardiac output to maintain oxygen delivery, resulting in increased cardiac dimensions and heart rate. Increased cardiac output and diastolic dysfunction cause abnormal loading of the pulmonary artery. Lung disease can exacerbate night-time hypoxia, a powerful stimulus for vasoconstriction. Hypersplenism is relatively common in the thalassemias and may necessitate spleen removal. Splenectomy may also be performed to lower blood transfusion requirements. However, the spleen plays a critically important role in removing hematologic debris from the cardiovascular system. As a result, splenectomy is a strong risk factor for intravascular thrombosis and pulmonary hypertension. Patients with thalassemia develop iron overload through increased iron absorption and transfusional therapy. Iron is toxic to all the endocrine glands that support the heart (Wood, 2009). The excess of iron from the blood transfusion requires intense iron chelation. Patients associated with a milder phenotype, as in β-thalassemia intermedia or non-transfusion dependent thalassemia, produce comparatively higher levels of hemoglobin and might require only sporadic transfusions. However, these patients exhibit increased iron absorption leading to severe iron overload and clinical sequelae. In addition, they are more prone to thrombotic-related complications than patients affected by β-thalassemia major (Rivella, 2015).

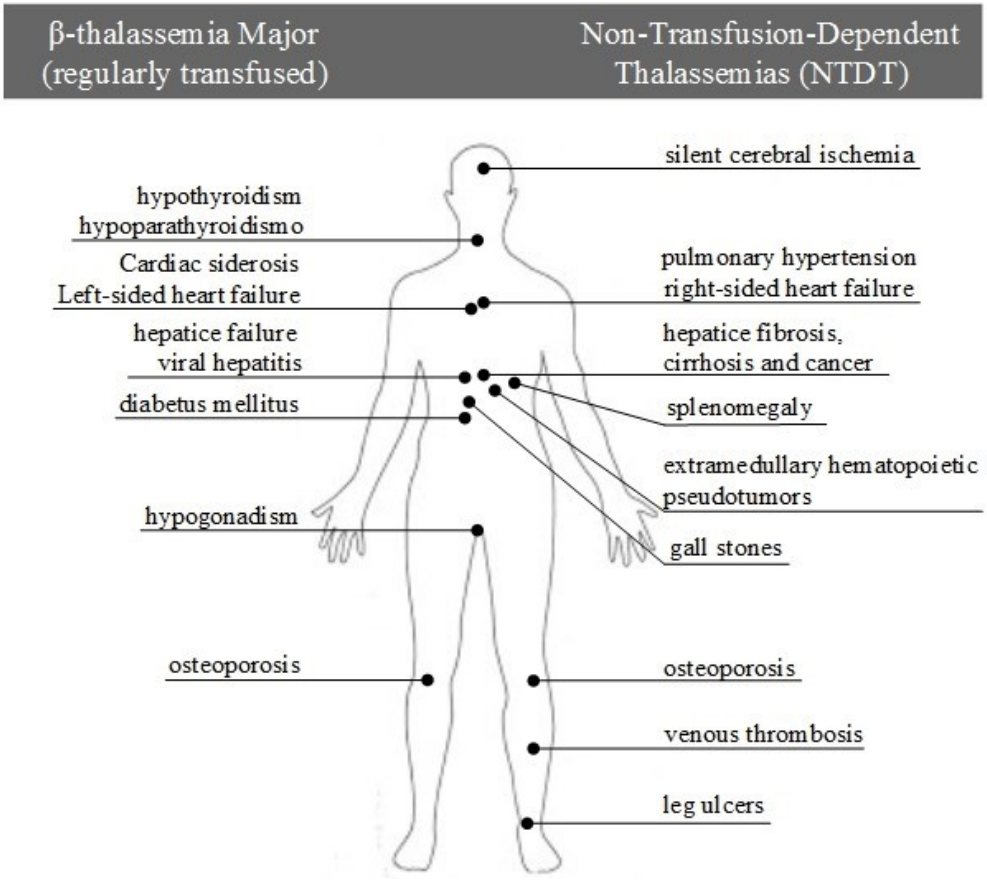


Figure 5. Possible clinical complications in β-thalassemias.

2.4. Treatment and management of β-thalassemia patients

- Transfusion therapy. The conventional treatment of patients affected by severe forms of β-thalassemia is based on regular blood transfusions. Red cell transfusions are given often enough, and in sufficient quantity, to maintain a hematocrit that is adequate to suppress hyperplasia, and to restore oxygen-carrying capacity (Benz EJ, 1996). Increasing evidence delineated the benefit of transfusion therapy in decreasing the incidence of complications as pulmonary hypertension and thromboembolic events.
- Iron chelation therapy. Iron-chelation therapy is required to maintain safe levels of body iron at all times. Adequate administration of parenteral desferrioxamine (DFO) reduces or prevents iron accumulation and iron-mediated organ damage, resulting in a consistent decrease of morbidity and mortality (Borgna-Pignatti et al., 1998). However, some patients may develop side effects of variable severity that affect compliance or sometimes result in cessation of treatment with DFO. Further chelating agents are:

Deferiprone (DFP) an orally active iron chelator, Desferrithiocin (DFT) a siderophore originally isolated from *Streptomyces antibioticus* in 1980 and then produced by chemical synthesis, Deferasirox (ICL670).

- Bone marrow transplantation. The only treatment that can currently be considered a cure for β-thalassemia is transplantation of hematopoietic stem-cells (BMT, bone marrow transplantation; CBT, cord blood transplantation) (Colah et al., 2010). In BMT, the donor is ideally a human leukocyte antigen (HLA)-identical sibling of the β-thalassemia patient to be transplanted. However, despite the availability of cord blood units and of matched donors has increased, about 60% of β-thalassemia patients lack a suitable donor (Lucarelli et al., 2012).
- Induction of fetal hemoglobin. Early observations of transient increases in fetal haemoglobin production in patients recovering after treatment with cytotoxic agents for leukaemia (Sheridan et al., 1976) suggest that acute erythroid expansion might be associated with fetal hemoglobin synthesis. It was reasoned that this phenomenon, possibly associated with perturbations of the cell kinetics of erythroid progenitors as a result of exposure to anti-leukaemic agents such as Hydroxyurea and Cytosine Arabinoside, might lead to a premature commitment to an erythroid maturation pathway with an increased likelihood of fetal haemoglobin production. Several laboratories have confirmed in independent *in vivo* trials that the treatment of β-thalassemia patients with the HbF-inducer Hydroxyurea (HU) leads to a clear improvement of the blood-intake requirements and even transfusion-independency in about 40-60% of the cases (Italia et al., 2009; Ansari et al., 2011).
- Gene therapy. Research efforts have been dedicated to the following major issues: highly efficient and stable transduction; targeting of the therapeutic vector to hematopoietic stem cells (HSCs); control of transgene expression (erythroid-specific, differentiation and stage-restricted, elevated, position-independent and sustained over time); low or absent genomic toxicity; selection of the transduced HSC in vivo; correction of the β-thalassemia phenotype in pre-clinical models, including transgenic mice; application of the knowledge on gene therapy for corrections of β-globin gene expression in iPS cells generated from adult cells of β-thalassemia patients. Clinical trials of gene therapy of β-thalassemia patients have been reported (Bank et al., 2005).

3. SICKLE CELL DISEASE

3.1. Characteristics

The first clinical description of abnormally elongated red blood cells in an anemic patient and the link with the clinical symptoms of what is now called sickle cell anemia (SCA) was published in 1910 (Herrick, 1910). This pathological condition is a result of a point single-mutation in the HBB gene that leads to the substitution of valine for glutamic acid at position 6 of the β-globin subunit (β^S). The defect results in a mutant form of hemoglobin called sickle haemoglobin (HbS), which polymerizes under low oxygen conditions (e.g. stress, hypoxia, or acidosis) and deforms red blood cells (Figure 6). These RBCs have a characteristic sickle (half-moon) shape and a reduced lifespan (from 120 days to 10–20 days).

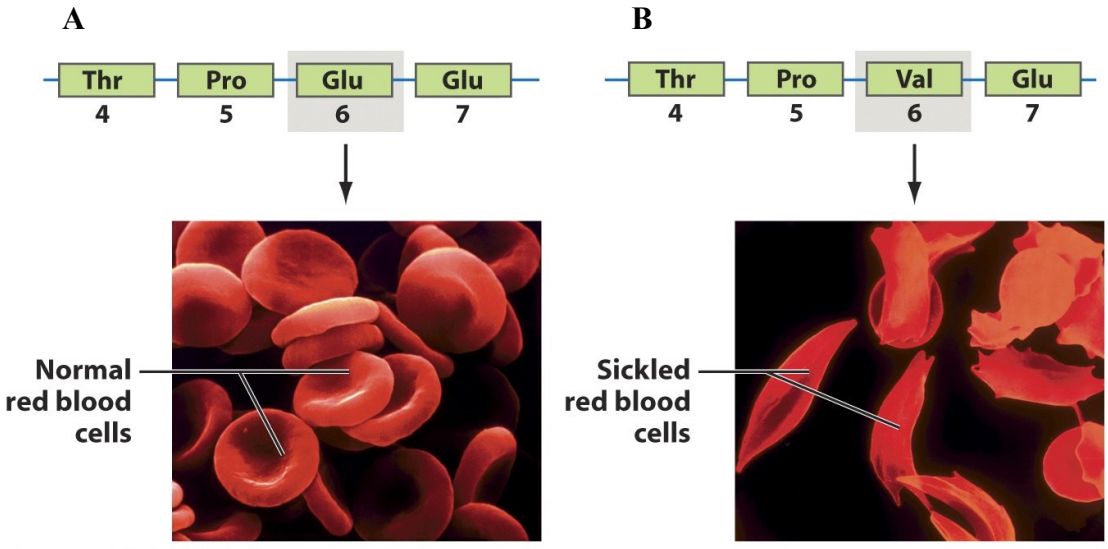


Figure 6. Representation of normal red blood cells (A) and sickle cell (B). The substitution of valine for glutamic acid at position 6 of the β-globin subunit is also depicted.

Homozygous inheritance of the β^S-mutation causes the most common form of this disease (>70% of SCD worldwide), usually referred to as either 'SCD SS' or as 'sickle cell anaemia' (SCA). However, other forms of SCD can also result from the inheritance of β^S in combination with a wide range of other HBB mutations, the two most common being a

second structural β-globin variant β^C (SCD SC) (Nagel et al., 2003) and one of the many β-thalassaemia mutations that lead to the reduced production of normal β-globin (SCD S/β-thalassaemia).

Heterozygous carrier do not exhibit clinical disease. This individuals are so strong protected from malaria that the global distribution and the frequency of the β^S-mutations now strongly reflects the historic incidence of death from malaria (Piel et al., 2010). Population migration during the last few hundreds of years had led to a substantial numbers of children with SCD are born in high-income countries, particularly in the larger cities in Europe and North America (Piel et al., 2014).

The altered sickle RBCs tend to adhere to each other causing vaso-occlusion and tissue hypoxia. Circulating free Hb released by damaged RBCs results in abnormal nitric oxide metabolism; moreover, inflammation, platelet activation, increased adhesion of RBCs to the vascular endothelium, and neutrophils activation occur. The clinical consequence, termed vaso-occlusive episodes (VOE) include pain crisis, respiratory insuffiency ("acute chest syndrome") and strokes (Manwani et al., 2013).

In low-income countries, where treatments are rarely available, the majority of children born with SCA die before the age of five years (Piel et al., 2014).

3.2. Clinical course

Intravascular haemolysis in SCD results from complement recognition of sickling-induced membrane changes, cell dehydration and direct membrane damage by rigid haemoglobin polymers. In order to compensate for the reduced oxygencarrying capacity, sickle cell patients have a hyperdynamic circulation, an expanded plasma volume, and develop dilated cardiomyopathy at an early age (Schnog et al., 2004). Pain (acute, chronic or neurophatic), in SCD patients, is caused by small vessel blockage and subsequent tissue infarction or organ impairment. Chronic pain may occur secondary to avascular necrosis and leg ulcers, or may be the result of persistent or frequently occurring acute painful episodes that are inadequately treated over time (Raphael et al., 2013). Vaso-occlusive episodes can occur throughout the body, including bones, muscles, mesentery, and other organs. Acute chest syndrome presents with clinical symptoms similar to pneumonia when VOEs affects pulmonary vasculature. Vaso-occlusion in the peripheral retina led to retinopathy, with the consequence of progressive neovascularisation and enlargement of

pre-existing capillaries occurs. Vitreous haemorrhage is a common complication and may result in blindness. Stroke is also a significant complication in SCD, with the potential for major morbidity and mortality (Strouse et al., 2009). The highest incidence is observed in the first decade of life, with a high recurrence rate. Both ischaemic and haemorrhagic stroke occur at all ages, even though ischaemic stroke occurs more frequently in the young, and haemorrhagic stroke is more frequent in patients in the third decade of life (Schnog et al., 2004). Asthma is also common in children with SCD, with a prevalence of 8–53%. Renal dysfunction in SCD patients is described as early as in infancy appearing as hyperfiltration, hypertrophy, and impaired urinary concentrating ability (Nath et al., 2015). Figure 7 reports all the possible complications in children and adults with SCD.

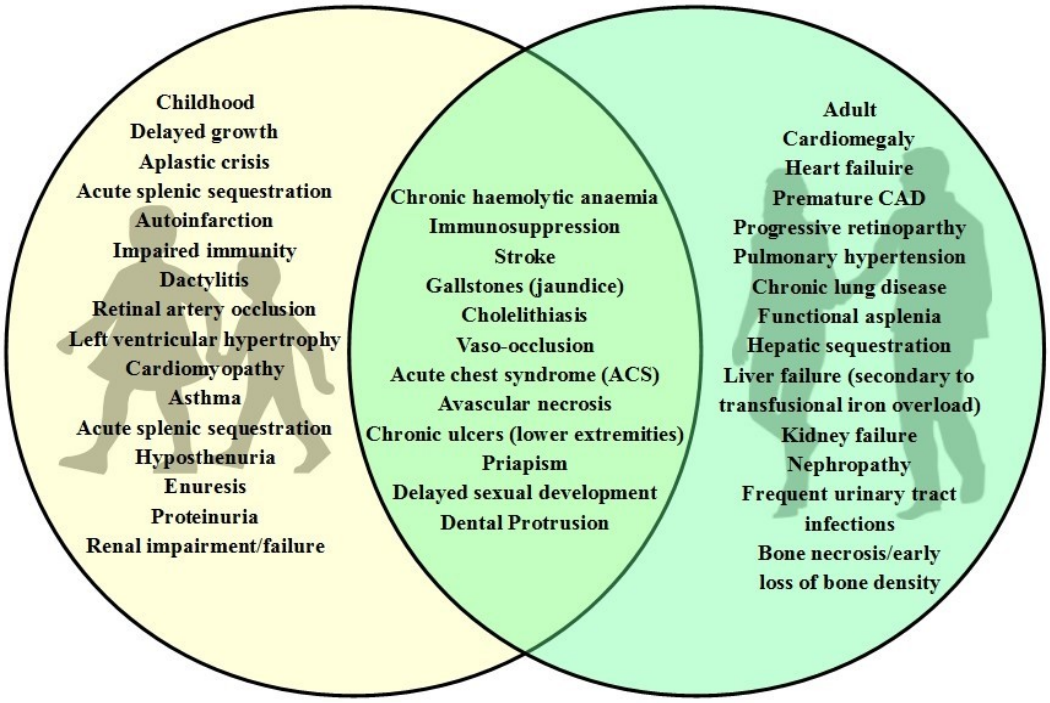


Figure 7. Complications of sickle cell anemia in children and adults.

3.3. Treatment and drugs

➢ Transfusion therapy. Chronic red cell transfusion programs aimed at reducing the HbS% below 30% in patients with stroke significantly reduce the risk of stroke recurrence

(Steinberg, 1999). However, chronic transfusions and exchange transfusions may lead to iron overload and iron deposition in organs (liver, heart, pituitary, and pancreas), with end-organ damage potentially occurring before the onset of symptoms.

- Chelation therapy. The aims of chelation therapy in SCD are similar to other forms of transfusional iron overload, namely to maintain body iron in tissues susceptible to iron-mediated damage at levels at which damage will not occur. Three iron chelators have been licensed in the United States and Europe for the treatment of iron overload and 2 of these, DFO and DFX, have been licensed for the treatment of iron overload in SCD. DFP has not been licensed specifically for the treatment of iron overload in SCD.

- Hydroxyurea. It is currently the only established preventative pharmacologic against painful crises. The mechanism of action is partly a result of the increased production of fetal hemoglobin, as well as decreased production of leukocytes and reticulocytes that may contribute to vaso-occlusion (Porter et al., 2013).

Haematopoietic stem cell transplantation (HSCT) has been shown to have an 85–90% success rate in certain paediatric patient groups. Adult patients require multi-disciplinary management of chronic conditions, such as stroke, cardiovascular complications (e.g. pulmonary hypertension), pulmonary complications, kidney failure, retinopathy, bone necrosis, and leg ulcers.

4. Hereditary persistence of fetal hemoglobin (HPFH)

Hereditary persistence of fetal hemoglobin (HPFH) is a descriptive term for a range of disorders with a genetically determined persistence of HbF production into adult life. The inherited persistent production of HbF in adult life belongs to two different categories according to the distribution of HbF within the red blood cells, which can be pancellular (homogeneous distribution) or heterocellular (heterogeneous distribution). Pancellular HPFH are caused either by deletion at variable extent of the β-globin cluster or by point mutation in the $^{G}\gamma$ or $^{A}\gamma$ promoter. Heterocellular HPFH are associated with quantitative trait loci linked or unlinked to the β-globin cluster 7. Variable increases in HbF levels have also been noted in patients with sickle cells anemia (SCA) and β-thalassemia. HbF reduces the propensity for HbS polymerization in sickle cells (Steinberg et al., 2009) and low levels of HbF have been associated with increased risk of brain infarcts in young children with

SCD (Wang et al., 2008). Infants with β-thalassemia only become symptomatic following the decrease in HbF production as the normal developmental fetal-to-adult hemoglobin switch occurs. More-recent clinical studies have substantiated the quantitative ameliorating effect of increased HbF production on the clinical course in a variety of individuals with β-thalassemia (Musallam et al., 2012; Galanello et al. 2009).

Three major HbF Quantitative Trait Loci (QTL), accounting for 20–50% of HbF variation, have been identified so far. The first, the so-called −158 C>T XmnI SNP (rs7482144), is located in the fetal ^{G}γ-globin gene promoter (Labie et al., 1985). The other two are located in the BCL11A gene and in the HBSB1L-cMYB inter-region, and are either involved directly in fetal gene silencing in adult life or in cell proliferation and differentiation (Uda et al., 2008). Some particular tag-SNPs in these regions are associated with high HbF levels in healthy adults, as well as in thalassemia and sickle cell disease (SCD) patients (Lettre et al., 2008; So et al., 2008).

Point mutations associated with HPFH are clustered in regions of the γ-globin gene promoters, where binding sites for erythroid-specific transcription factors are present. A variety of transcription factors played roles in globin gene regulation, including GATA1, KLF1 (EKLF), and SCL/ TAL1 (Cantor et al., 2002). Within the γ-promoter, there are a single CACCC box, two CAAT box and a canonical TATA box. The transcription factors FKLF and FKLF2 were found to bind to a CACCC site at position -145 of the γ-promoter (Asano et al., 1999). Genetic association studies have identified sequence variants in the gene BCL11A that influence HbF levels. BCL11A does not occupy the gamma-globin promoter but binds to the LCR as well as intergenic regions in the cluster associated with γ-globin repression. BCL11A form complexes with GATA1 and FOG1 and additionally with the NuRD chromatin remodeling complex (Sankaran et al., 2008). Furthermore, BCL11A physically interacts with SOX6 and collaborates with this transcription factor in the repression of fetal globin gene expression (Figure 8).

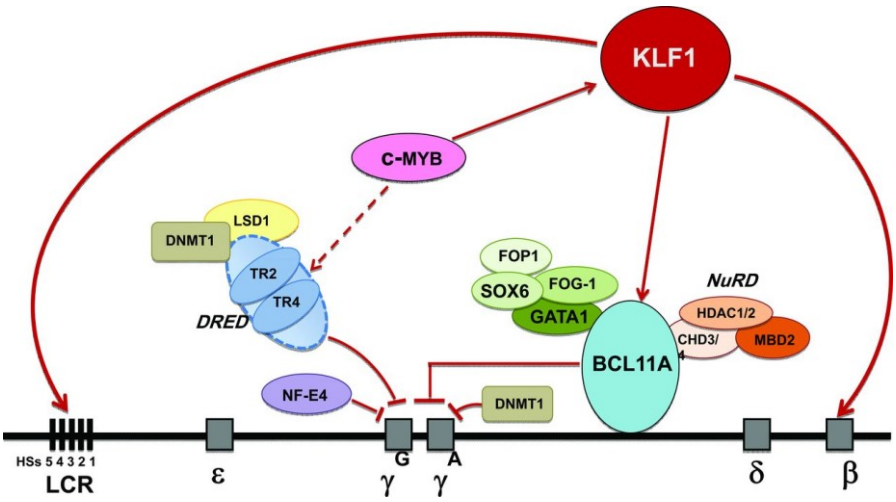


Figure 8. A current model of fetal globin gene silencing. Targets identified in the emerging network of HbF regulation include the KLF1, BCL11A, MYB and the TR2/TR4 nuclear receptors. BCL11A interacts with the GATA1, FOG1, and SOX6 erythroid transcription factors and with the NuRD deacetylase and remodeling complex to promote suppression of γ-globin gene expression.

MYB, which encodes the c-MYB transcription factor, is a key regulator of hematopoiesis and erythropoiesis. Direct knockdown of MYB in primary adult erythroid progenitors resulted in dramatic increases in γ-globin production (Sankaran et al., 2011). A low MYB environment favors accelerated erythropoietic differentiation, leading to the release of early erythroid progenitors that are still synthesizing predominantly HbF. Bianchi et al. have reported that MYB activates KLF-1 and TR2/TR4 in human erythroid cells (Bianchi et al., 2010).

Recently LYAR was found to binds to the DNA region corresponding to 5'UTR of the γ-globin gene and to participate in silencing human fetal hemoglobin gene expression (Ju et al., 2014).

AIM AND THESIS OUTLOOK

In β-thalassemia and sickle cell anemia (SCD), the survival of fetal hemoglobin (HbF) production in significant amount can reduce the severity of the clinical course and reactivation of γ-globin gene expression in adulthood has proven to be one of the best strategies to ameliorate the symptoms of patients with β-hemoglobinopaties. Hydroxyurea is currently the only US Food and Drug Administration–approved medication to induce fetal hemoglobin, although treatments with HU generate sufficient levels of HbF in only half of patients (Steinberg et al., 1997) and side effects including leukopenia and neutropenia are frequently reported. Therefore, novel therapeutic inducers must be identified in order to develop a personalized treatment of patients with β-thalassemia and sickle cell anemia.

Part I of this PhD thesis focuses on the characterization of novel HbF inducers. The availability of new treatments may be offered by the use of drugs already approved for other indications, since pharmacokinetics and adverse event profile in patients have been already assessed. Rapamycin (as Sirolimus), an immunosuppressant agent approved by the FDA for prevention of acute rejection in renal transplant recipients, was found to induce γ-globin gene expression in human erythroleukemia cell line and erythroid precursors cells (ErPCs) from patients with β-thalassemia (Mischiati et al., 2004; Fibach et al., 2006). Our aim was to validate and further investigate the preliminary results obtained in a wider number of patients with β-thalassemia. Resveratrol, a natural polyphenolic compound largely used in nutraceutical products for its antioxidant property, was described as a HbF inducers in erythroid precursors cells (Rodrigue et al., 2001; Fibach et al., 2012). At the moment, no toxic or adverse effects of Resveratrol in humans by administering a single dose up to 5 grams have been described. In collaboration with Dott. Claudio Favre we have evaluate the effects of the oral administration of a Resveratrol-nutraceutical in β-thalassemia patients. The study was carried out at the Pediatric Oncohematology Unit, Santa Chiara Hospital (Pisa) and patients were recruited following all the ethical requirements and the approval of the Ethical Committees of the Hospital. The induction of γ-globin gene expression and HbF production during *in vivo* administration of Resveratrol has been evaluated in ErPCs isolated from peripheral blood. A study published in 2003 reports that Angelicin, a furanocoumarin is able to induce fetal hemoglobin production in human erythroid progenitors (Lampronti et al., 2003). In order to ameliorate biological activity and to reduce possible undesirable effects (e.g. genotoxicity), other Angelicin

analogues have been synthesized by Prof. Adriana Chillin (Department of Pharmaceutical and Pharmacological Sciences of Padua). We have tested 38 Angelicin analogues in human erythroleukemia cells at first and in erythroid precursors cells isolated from peripheral blood of β-thalassemia patients.

Severe clinical complications of β-thalassemia and sickle cell anemia may occur due to the accumulation of free α-globins or to the production of defective β-globin, respectively. A number of case-control and cohort studies have demonstrated that a natural reduction in α-globin chain output, resulting from coinherited α-thalassemia, is beneficial in patients with β-thalassemia. Therefore, reducing α-globin chains in patients with β-thalassemia is a potential pathway to develop new therapies (Mettananda et al., 2015). Molecular biology based methods enabling efficient inhibition of accumulation of this defective globin can be of great interest. Peptide nucleic acids (PNAs) have been largely used for their remarkable hybridization properties and their various applications. Moreover their high stability and their resistance to nuclease and protease allow the use of PNAs to regulate gene expression both *in vivo* than *in vitro*.

Part II of this PhD thesis is concerned about the potential therapeutic application of peptide nucleic acids (PNAs) in erythroid cells. In order to obtain a fast and efficient delivery in erythroid cells without strong antiproliferative effects, we have evaluated the efficiency of a liposomal formulation of a PNA anti-miR-210 in human erythroleukemia cell line. Moreover, an antisense PNA was designed in order to target murine β-globin mRNA and tested in murine erythroleukemia cell line. Finally, we have examined the ability of a PNA against sickle β-globin mRNA in erythroid precursors cells isolated from SCD patients.

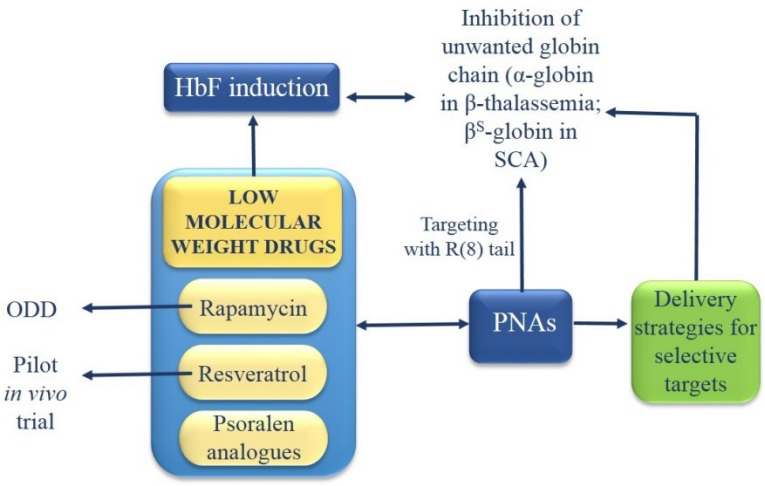


Figure 9. Flow chart

Part I:

Characterization of novel fetal hemoglobin inducers

1. RAPAMYCIN

Rapamycin (Figure 10) is a lipophilic macrolide, also called Sirolimus, isolated in 1975 from a strain of *Streptomyces hygroscopicus* found in a soil sample from Easter Island (known by the inhabitants as Rapa Nui) (Sehgal, 2003).

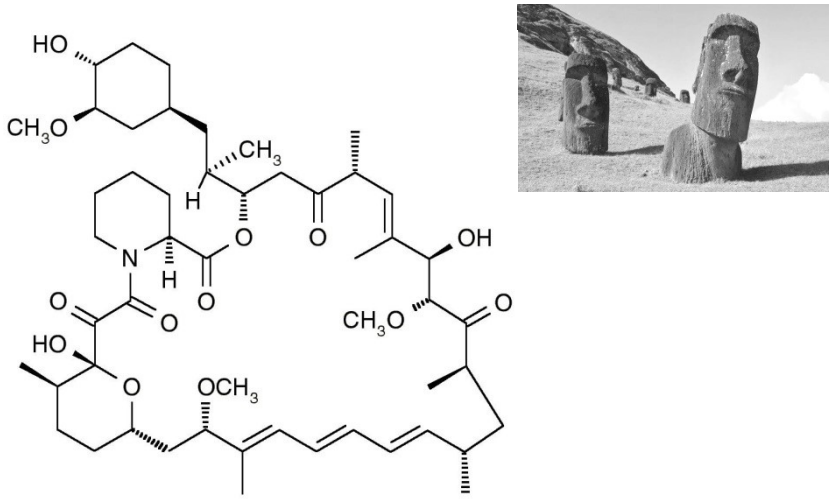


Figure 10. Molecular structure of Rapamycin, a 31-membered macrocyclic polyketide, first isolated from samples of *Streptomyces hygroscopicus* found on Easter Island.

1.1. INTRODUCTION

1.1.1. Biosynthesis

The biosynthesis of Rapamycin has been extensively studied, but many elements of the biosynthetic machinery remain obscure. The amount of Rapamycin produced from *S. hygroscopicus* depends on essential nutrients available. Different sources of nitrogen sources can largely impact the production of Rapamycin, but it has been reported that ammonium sulfate administered at 40 mM resulted in the highest yield of the natural product in comparison with five other non-amino acid nitrogen compounds (Lee et al., 1997). Another study by Cheng et al. demonstrated that L-lysine must be present for Rapamycin production and that increases in lysine resulted in high yields of Rapamycin (Cheng et al., 1995). In *Streptomyces hygroscopicus*, three large polyketide synthase genes

are responsible for the biosynthesis of Rapamycin. These genes encode for 14 enzyme-containing modules, each having a specific role in elongating the Rapamycin polyketide. The three polyketide synthase genes, rapA, rapB, and rapC, encode the multienzyme polyketide synthases Raps1, Raps2, and Raps3, respectively (Schwecke et al., 1995). Rasp1 contains a loading zone for the starter unit 4,5-dihydroxycyclohex-1-enecarboxylic acid, derived from shikimate (Figure 11) (Gregory et al., 2004). Three propionate units and one acetate unit are added with various oxidation states and the resulting polyketide of Raps1 subsequently enters Raps2. Raps2 adds three acetate units and three propionate units. The resulting fragment is transferred onto module 11 of Raps3 where four more successive rounds of elongation take place Raps3 adds three acetate units and a propionate unit. At this point, pre-rapamycin has a total of seven acetate and seven propionate units in addition to the shikimate derivative.

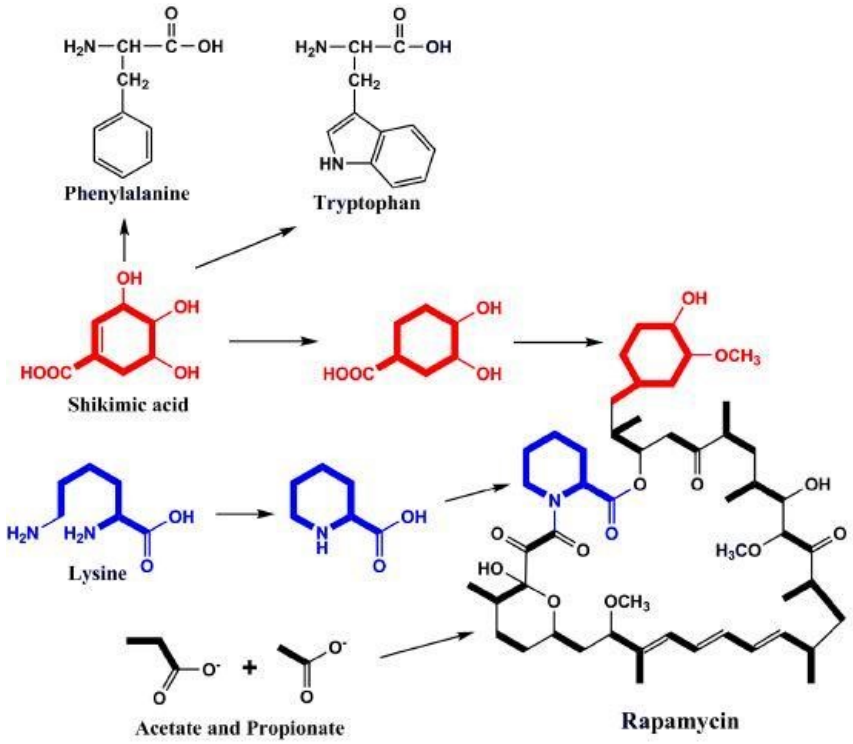


Figure 11. Rapamycin biosynthetic pathway. Possible metabolic pathways related to the precursors of Rapamycin biosynthesis are specified (From: Zhu X et al., *Biotechnol Bioeng* 2010).

Afterwards, the pipecolate-incorporating enzyme (PIE) (produced by the RapP gene adjacent to Raps3), binds to an activated L-pipecolate through a thioester linkage. PIE then

catalyzes the transfer of the polyketide onto the amino group of pipecolate by a nucleophilic attack of the imino nitrogen of pipecolate to the carbonyl group of the last added acetate unit. Subsequent attack by C-34 hydroxyl group forms the "macrolactam ring" (Konig et al., 1997). Further oxidations and methylations occur to produce the end product, Rapamycin. Several chemical synthesis have been described, but the first was developed by Nicolaou et al in 1993 (Nicolaou et al., 1993).

1.1.2. Biological activity and mechanism of action

Rapamycin has generated sustained interest from scientist because of its therapeutical potential. Sirolimus is above all an immunosuppressive agent (Dumont et al., 1996), in fact it has received approval from the US Food and Drug Administration for marketing as an agent for the prevention of acute rejection in renal transplant recipients. In addition, Rapamycin displayed activity against various diseases including, but not limited to, cancer (Huang et al., 2001; Shi et al., 2008; Kobayashi et al., 2007), HIV (Nicoletti et al., 2009), Parkinson (Tain et al., 2009). Rapamycin and several of its structural derivatives [Temsirolimus (Wyeth Pharmaceuticals), Everolimus (Novartis Pharmaceuticals), and AP-23573 (Ariad Pharmaceuticals)] are currently under robust and encouraging investigational use in phase I and II clinical trials for a variety of cancers (Dancey, 2005). The biological responses of Rapamycin appear to mostly depend on mTOR, which is a 290-kDa (2,549–amino acid) serine/threonine kinase with many functional and protein-binding domains. The kinase domain of mTOR is located in its COOH-terminal region between a FATC domain and a FKBP-Rapamycin binding domain. Indeed, Rapamycin inhibits the highly conserved protein kinase target of Rapamycin (TOR) by forming a complex with immunophilin FKBP12 (12 kDa FK506-binding protein; also known as FKBP1A), which then binds directly to mammalian TOR complex 1 (mTORC1) but not to mTORC2 (Benjamin et al., 2011). Rapamycin-FKBP12 inhibits the kinase by directly blocking substrate recruitment and by further restricting active-site access. One of the downstream targets of mTOR is the eukaryotic initiation factor 4E binding protein (4E-BP1), whose phosphorylation by mTOR stimulates protein translation through releasing eukaryotic initiation factor 4E from 4E-BP1 (Hara et al., 1997). Regulation of protein synthesis via mTOR also occurs through phosphorylation of p70 S6 kinase, a key regulator of cell growth, which phosphorylates the S6 40S ribosomal subunit. The m-TOR dependent

phosphorylation of both 4E-BP1 and p70 kinase is regulated by the small G protein Ras homologue enriched in brain (Rheb) (Tee et al., 2003). Although mTORC2 was initially described as resistant to the effects of Rapamycin, prolonged treatment with high doses of Rapamycin is also able to inhibit mTORC2 activity by impeding the binding and subsequent assembly of mTORC2-specific components mSIN1 and RICTOR (Bové et al., 2011).

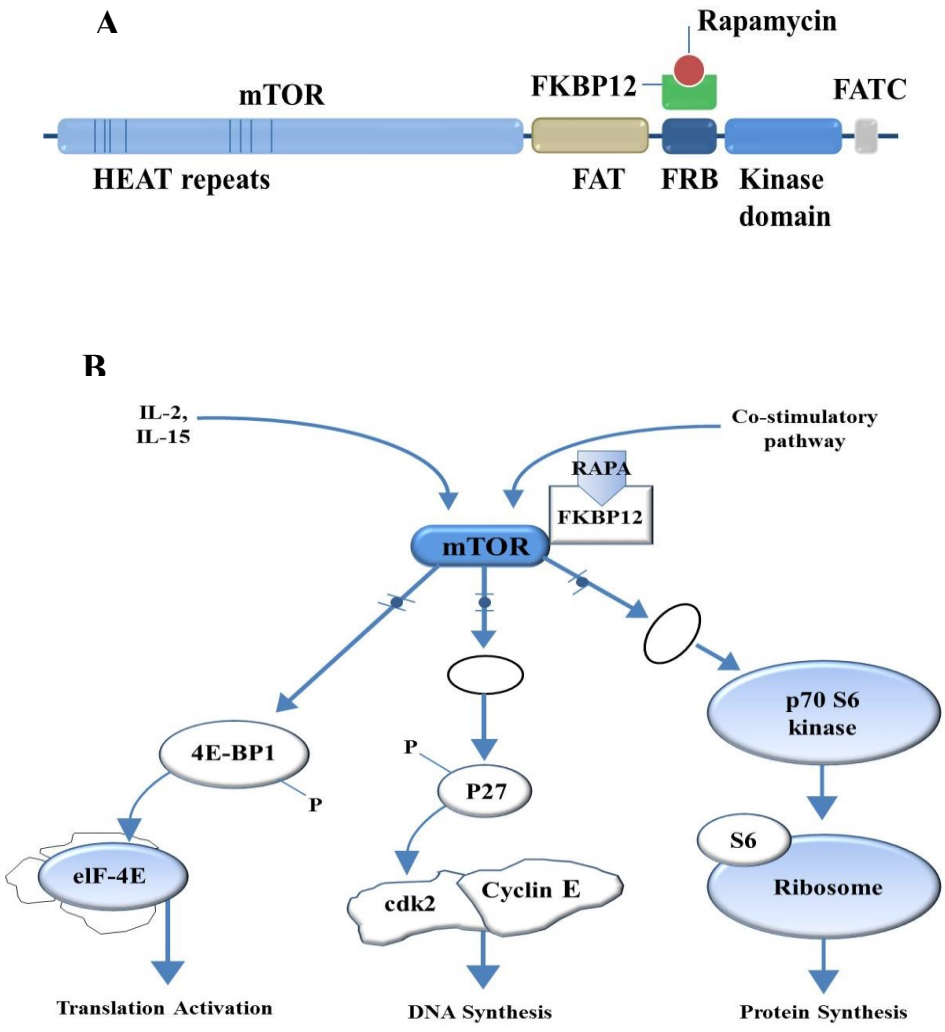


Figure 12. (A) Schematic depiction of the main features of mTOR. The HEAT repeats likely mediate interactions between mTOR and some of its partners, FKBP-Rapamycin binding domain, FAT and FATC domains. **(B) Proposed mechanism of action for Rapamycin.** Rapa inhibits phosphorylation of 4E-BP1, preventing release of eIF-4E and initiation of translation; P27-mediated activation of cdk2-cyclin E and synthesis of proteins important for cell cycle progression and p70S6 kinase activation, limiting ribosomal protein S6 phosphorylation and reducing synthesis of ribosomal/translational proteins.

1.1.3. Preliminary studies on erythroid differentiation

The interest for Sirolimus (or Rapamycin) as a potential inducers of fetal hemoglobin was triggered by the discovery of its ability to induce the differentiation of human myeloid leukemia HL-60, ML-1, K562 cells (Yamamoto-Yamaguchi et al., 2001) and J2E cells (Jaster et al., 1996).

Hence, differentiation of K562 cell line was found to be associated with a sharp increase in the production of γ-globin mRNA (Mischiati et al., 2004). Interestingly, this increase was not observed in cells treated with tacrolimus (FK506) or ascomycin, two immunophillins that display a similar molecular structure and targets, but unable to modulate FRAP/mTOR (Saunders et al., 2001). This data suggested that FRAP/mTOR, and not FKBP12, is implicated in the complex pathway leading to erythroid differentiation. Sirolimus was then tested in erythroid precursors cells isolated from peripheral blood of β-thalassemia patients. The results indicated that Rapamycin increases fetal hemoglobin (HbF), even if the starting levels of this protein were sharply different. The inducing effects of Rapamycin were selective for γ-globin mRNA accumulation (Figure 13), being only minor for β-globin and none for α-globin mRNAs. A strong correlation between the increase in the HbF and the increase in γ-globin mRNA content was highlighted (Fibach et al., 2006). More recently, another research demonstrated an increase in vitro of γ-globin mRNA expression in 15 sickle cell disease and 14 β-thalassemic patients and a corresponding HbF increase (Pecoraro et al., 2015).

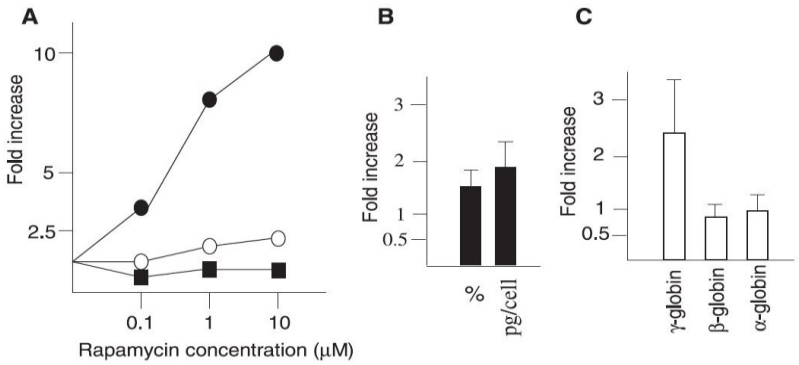


Figure 13. Effects of Rapamycin on globin mRNAs and haemoglobins of cultured erythroid progenitors. A. Fold increase of α-globin (black boxes), β-globin (white circles) and γ-globin (black circles) mRNAs in erythroid progenitor cells treated with increasing concentrations of Rapamycin. **B, C**. Correlation between increase of HbF and accumulation of globin mRNAs. Fold increase in HbF (% of total Hb and pg/cell) (**B**) and α-, β- and γ-globin mRNA (**C**) following treatment of erythroid precursor cells from b-thalassaemia patients. (From: Fibach et al., *Eur J Haematol* 2006)

As for the mechanism of action, Bianchi et al found that Sirolimus-mediated erythroid induction is associated with a hypophosphorylation of α-p-S6 ribosomal protein and with hyper-phosphorylation of 4E-BP-1 (Figure 14). Furthermore, it was demonstrated that inactivation of both 4E-BP1 and p70-S6K are sufficient steps, since we induced differentiation when these molecular events were simultaneously produced by a mixture of drugs involved in downstream alterations of the FRAP-mTOR signal transduction pathway (Bianchi et al., 2015).

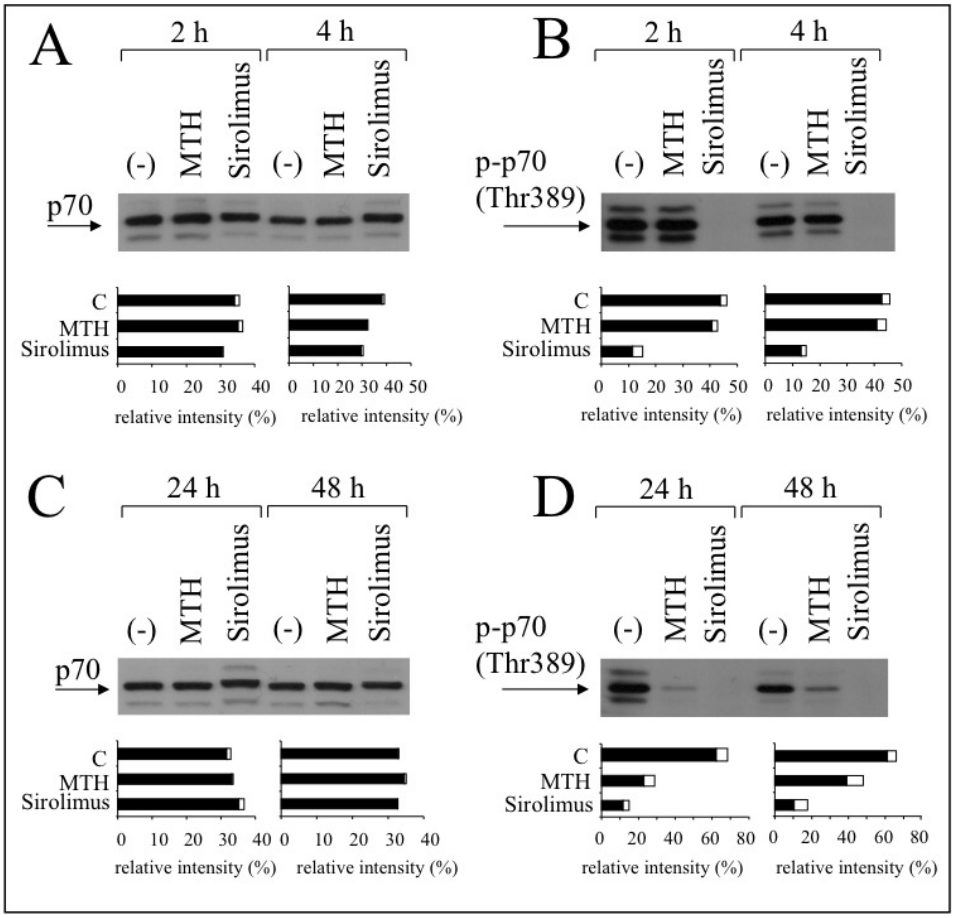


Figure 14. Comparison of the effects of Mithramycin (MTH) to those of Sirolimus on phosphorylation of p70S6Kinase, a mTOR-C1 target. (**A,C**) K562 cells were treated with 30 nM MTH and 1 μM Sirolimus and analyzed after 2, 4, 24, 48 h by Western blotting using monoclonal antibodies against p70S6Kinase (**A,C**) and against p-p70S6K Thr389 phosphorylation site (**B,D**); (-) indicates untreated cells. Hyposphorilation of p70S6Kinase is evident even after very short exposure to Sirolimus (2 hours) (From: Bianchi et al., *PLoS-ONE*, 2015).

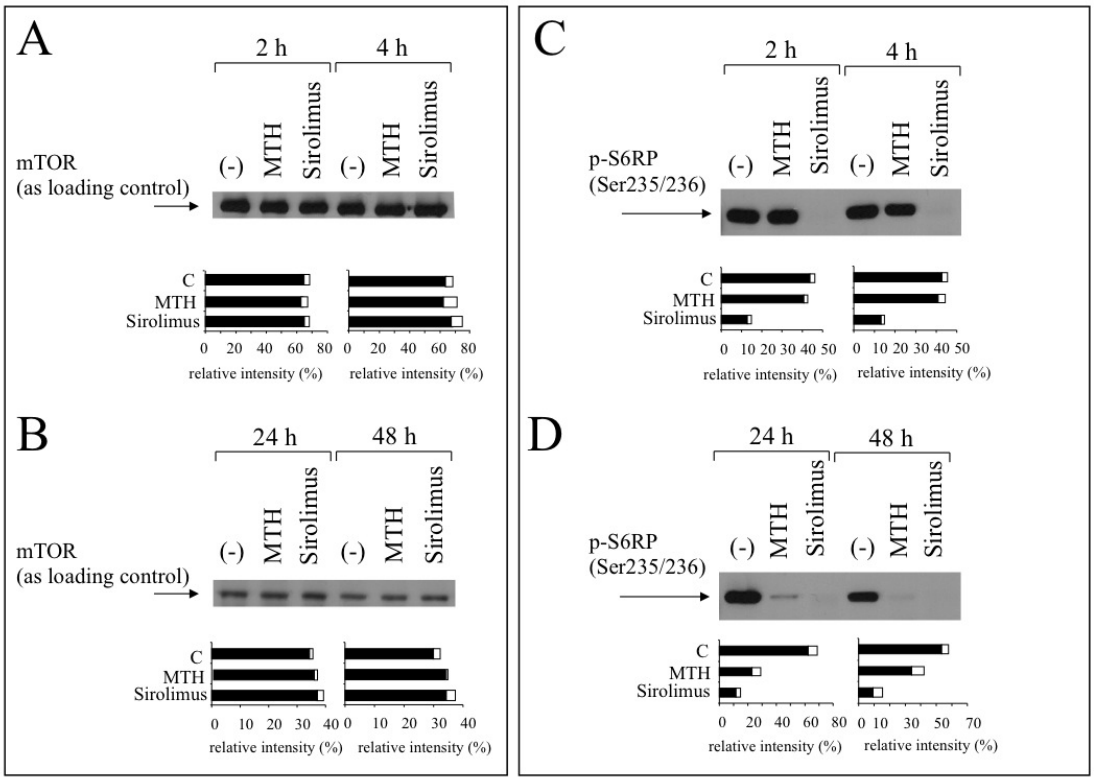


Figure 15. Western blotting was performed using monoclonal antibodies against mTOR and p-S6RP Ser235/236 phosphorylation site. The lower part of each panel reports the graphs obtained by densitometric analysis of the films. Normalized intensity values are expressed as a percentage and mean (black) and standard deviation (white) from three independent experiments are reported. Hyposphorilation of p-S6RP is evident even after very short exposure to Sirolimus (2 hours) (From: Bianchi et al., *PloS-ONE*, 2015)

1.2. MATERIALS AND METHODS

1.2.1. Culture of human K562 erythroleukemia cells

The human K562 erythroleukemia cells, isolated and characterized by Lozzio and Lozzio (Lozzio et al., 1975) from a patient with chronic myelogenous leukemia (CML) in blast crisis, has been extensively employed as a very useful in vitro model to study the molecular mechanism(s) regulating the expression of embryonic and fetal human globin genes (Rutherford et al., 1981) as well as to determine the therapeutic potential of new differentiation-inducing compounds. K562 cells growth in culture as single, undifferentiated, cells in suspension, with low production of hemoglobins. When stimulated by various agents, they are able to differentiate in erythroid precursors cells, as

demonstrated by Rutherford et al. in 1979 (Rutherford et al., 1979). Erythroid differentiation of K562 cells is associated with an increase of production of both haemoglobin (Hb) Gower 1 and Hb Portland (Bianchi et al., 2001). K562 cells are usually cultured in humidified atmosphere of 5% CO_2, in Roswell Park Memorial Institute RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 50 units/ml penicillin (Lonza, Verviers, Belgium) and 50 μg/ml streptomycin (Lonza, Verviers, Belgium).

1.2.2. Culture of erythroid precursors isolated from peripheral blood of β-thalassemia patients

Large cultures of relatively pure and synchronized erythroid cell population can be obtained from peripheral blood and compounds can be added on different days when the culture consists of cells at specific stages of maturation. In the procedure developed by Fibach et al. (Fibach et al., 1989), the culture is divided into two phases. After obtaining informed consent, peripheral blood samples are collected from healthy donors and from patients with β-thalassemia (thanks to the collaboration between Ferrara University and St. Anna Hospital). Monuclear cells are isolated by Ficoll-Hypaque (Lympholyte H; Cedarlane, Burlington, NC, USA) density gradient centrifugation and then are seeded in a phase I medium culture, that include minimum essential medium with alpha modification (α-MEM; Sigma, Aldrich, St. Louis, Missouri, USA) supplement with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 1 μg/ml cyclosporine A (Sigma, Aldrich, St. Louis, Missouri, USA) and 10% conditioned medium from the 5637 bladder carcinome cell line. The cultures are incubated at 37°C, under an atmosphere of 5% CO_2 in air, with extra humidity for 7 days. Afterwards, the non-adherent cells are harvested, washed with phosphate buffered saline (PBS; Gibco, Life Technologies, Monza MB, Italy) and transferred in the phase II medium culture composed of α-MEM, 30% FBS, 1% deionized bovine serum albumin (BSA; Sigma, Aldrich, St. Louis, Missouri, USA), 10–5 M β-mercaptoethanol (β-ME; Sigma, Aldrich, St. Louis, Missouri, USA), 2 mM L-glutamine (Lonza, Verviers, Belgium), 10^{-6} M dexamethasone (Sigma, Aldrich, St. Louis, Missouri, USA), 1 U/mL human recombinant erythropoietin (EPO; Tebu-bio, Magenta, MI, Italy) and 10 ng/mL Stem Cell Factor (SCF, PeproTech EC Ltd, London, England) in 10 mM acetic acid solubilized (Fibach et al., 1989; Fibach et al., 1991). Compounds are usually

added on day 7 of phase II and the treatment lasts 5 days, after which RNA is extracted and HPLC analysis of lysed cells is performed.

1.2.3. Cell proliferation assay

Cells were counted with the BECKMAN COULTER® Z2 (Beckman, Pasadena, California, USA), in the proper range (8-20 μm for K562 cells and 6-12 μm for erythroid precursors), adding 10 mL of physiological water to 50 μL of cell suspension in a little becker (1:200 dilution). In order to obtain the data of cells number/mL, results should be multiply by 400 (considering the instrument volume metering 0.5 mL).

1.2.4. Benzidine assay: evaluation of erythroid differentiation

Cells containing heme or hemoglobin were detected using a specific reaction with a benzidin/ hydrogen peroxide solution (0.2% benzidine in 5mol/l glacial acetic acid, 10% H_2O_2) as reported elsewhere (Gambari et al., 1984). Cells suspension is diluited 1:1 with the benzidine solution at the time of analysis and blue stained cells are counted under an optic microscope. The number of blue positive cells to benzidine (B^+) are expressed as as percentage.

1.2.5. RNA isolation

The total cellular RNA was extracted by TRIZOL® Reagent (Sigma-Aldrich, St.Louis, Missouri, USA). All reagents and materials used were RNase-free. After 5 days of treatment with the compounds, erythroid precursors cells were centrifuged at 1,200 rpm for 10 minutes at 4°C, washed in 1X PBS, re-centrifuged and then lysed with 1 ml TRIZOL® Reagent. The homogenate was incubated 5 minutes at room temperature and 200 μl of chloroform were added; the samples were shaken vigorously for 15 second, incubated for 5 minutes at room temperature and finally centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred into a clean tube and 500 μl of

isopropanol was added. The RNAs were and incubated for 10 minute at room temperature (25°C). After that, the samples were centrifuged at 12,000 rpm for 10 minutes at 4°C; the isolated RNA was precipitated in 2 volumes of absolute ethanol and stored at -80°C, washed once with cold 75% ethanol, dried and dissolved in 10 µl of diethylpyrocarbonate (DEPC)-treated water before use (Sambrook et al., 1989) and conserved at -80°C.

1.2.6. RNA quantification

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. The concentration is obtained by the equation:

g/ml = ODx40xDIL

where OD is the value read from the instrument, 40 is the correction coefficient for reading the RNA at the spectrophotometer (according to the Lambert-Beer law) and DIL is the dilution factor. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260 =1= 40 µg/ml). This relation is valid only for measurements in water.

1.2.7. RNA electrophoresis on agarose gel

The 1% agarose gel is prepared by dissolving 1 g of agarose powder in 100 ml of TAE 1x (obtained by dilution from 50x TAE = 2 M Tris-HCl, 0.05 M EDTA pH = 8.0 and 5.71% acid Acetic 99.8%); EtBr 10 g/ml was added to the solution.

1.2.8. Reverse transcription reaction- Random Hexamer

Reverse transcription of 500 ng of total RNA was performed using with the TaqMan® Reverse Transcription Reagents PCR kit (Applied Biosystems). RNA was incubated with 2,5 µM Random Hexamers at 25°C for 10 minutes and then immediately cooled to 4°C. After that, the RT reaction solution, prepared considering a final volume of 50 µl, progressively adding the following reagents: 1X TaqMan RT Buffer (10X), 5,5 mM $MgCl_2$ (25 mM), deoxyNTPs Mixture, 0,4 U/µl RNase Inhibitor and 1,25 U/µl

MultiScribeTM Reverse Transcriptase (50 U/µl) was added. To perform the reverse transcription reaction, the samples were incubated for 30 minutes at 48°C, to allow the extension, and at 95°C for 5 minute to inactivate the enzyme. After thermal cycling, cDNA were stored at -80°C.

1.2.9. Real Time quantitative-PCR analysis

All RT reactions, including no-template controls and RT-minus controls, was performed in duplicate using the iQ™5 Multicolor Real-Time PCR Detection System (BIORAD, Hercules, California, USA). Relative expression was calculated using the comparative cycle threshold method and as reference gene RPL13A (60S Ribosomal protein L13a) was used to normalize all RNA samples, because it is equally expressed in the assayed samples as previously assessed. RT-qPCR assay was carried out using gene-specific fluorescently labeled probes (Eurofins MWG Operon, Ebersberg, Germany). The nucleotide sequences used for real-time qPCR analysis of α-, β- and γ-globin mRNAs are here reported:

Table 2. Sequences of primers and probes used in quantitative PCR reactions multiplex.

PRIMER-PROBE	SEQUENCE
Primer forward α-globin	5'-CGACAAGACCAACGTCAAGG-3'
Primer reverse α-globin	5'-GGTCTTGGTGGTGGGGAAG-3'
Probe α-globin	5'-**HEX**-ACATCCTCTCCAGGGCCTCCG-**BHQ**-3'
Primer forward β-globin	5'-GGGCACCTTTGCCACAC-3'
Primer reverse β-globin	5'-GGTGAATTCTTTGCCAAAGTGAT-3'
Probe β-globin	5'-**Texas Red**-ACGTTGCCCAGGAGCCTGAAG-**BHQ**-3'
Primer forward γ-globin	5'-TGACAAGCTGCATGTGGATC-3'
Primer reverse γ-globin	5'-TTCTTTGCCGAAATGGATTGC-3'
Probe γ-globin	5'-**FAM**-TCACCAGCACATTTCCCAGGAGC-**BHQ**-3'
Primer forward RPL13A	5'-GGCAATTTCTACAGAAACAAGTTG-3'
Primer reverse RPL13A	5'-GTTTTGTGGGGCAGCATACC-3'
Probe RPL13A	5'-**CY5**-CGCACGGTCCGCCAGAAGAT-**BHQ**-3'

1.2.10. High performance liquid chromatography (HPLC) analysis

Human erythroid precursor cells were harvested, counted (ZF Coulter Counter, Coulter Electronics, Hialeah, FL, USA), washed once with PBS, and pellets were lysed in water by freeze and thaw cycles and recovered by centrifugation at 14,000g for 30 min and 4 °C. Hb proteins present in the lysates were separated by cation-exchange HPLC, utilizing a Beckman Coulter instrument System Gold 126 Solvent Module-166 Detector. Hemoglobins were separated using a PolyCAT-A column, samples were eluted in a solvent gradient utilizing aqueous sodium chloride–BisTris–KCN buffers and detection was performed at 415 nm. The standard controls were the purified HbA (SIGMA, St Louis, MO, USA) and HbF (Alpha Wassermann, Milano, Italy).

1.3. RESULTS

1.3.1. Patient characterization

Patients were recruited following all the ethical requirements and the approval of the Ethical Committees of Ferrara Hospital. Blood samples were collected from 33 patients β-thalassemia patients after signature of the informed consent form. The genotype of all these patients was obtained, and summary of the composition of the samples is reported in Figure 16, which shows that the most frequent genotypes are $\beta^0$39/$\beta^0$39 (8 patients) and $\beta^0$39/β^+IVSI-110 (7 patients). A total of 39 *in vitro* treatment with Rapamycin were carried out, following the two-phase liquid culture method (called protocol A) described in the section “Materials and Methods” of this PhD thesis.

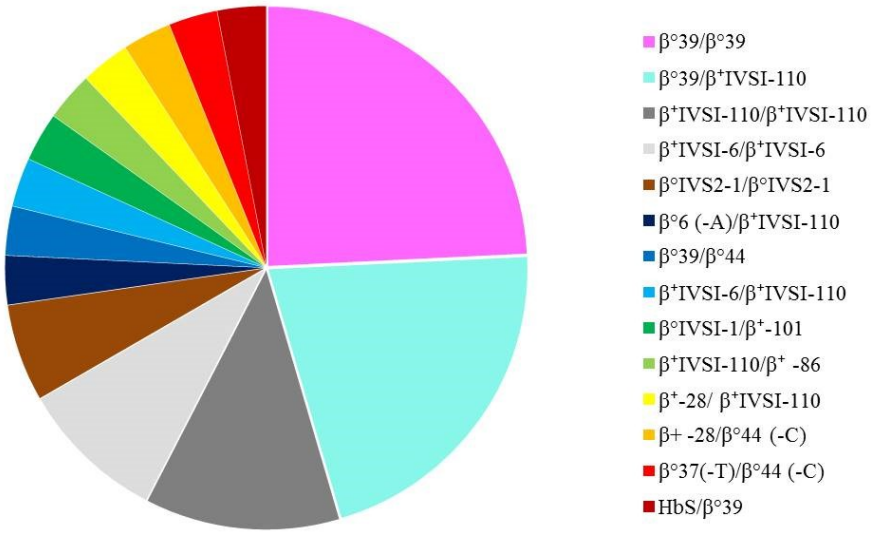


Figure 16. Genotypes of the patients recruited for the study.

1.3.2. Effects of Rapamycin on γ-globin gene expression

The distribution of induction obtained after the treatment of ErPC cultured with the two-phase method (protocol A) was compared to preliminary results obtained with the protocol C. Figure 17 shows the fold increase of γ-globin mRNA in Rapamycin-treated ErPCs when protocol A and C are used. Rapamycin induction of γ-globin mRNA is evident in both cases.

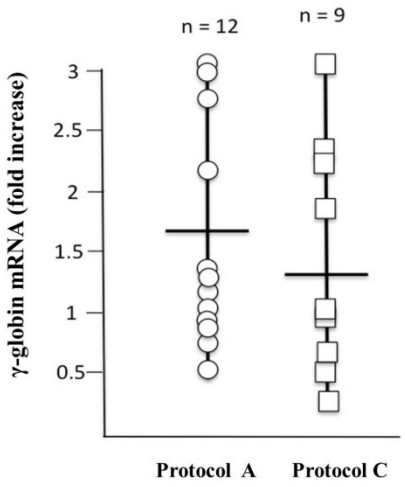


Figure 17. Increase of γ-globin mRNA following Rapamycin treatment of ErPC from β-thalassemia patients. The distribution of the results obtained following the protocol A (**left**) are compared with those of the protocol C (**right**), as indicated.

1.3.3. Relationship between the induction of γ-globin gene expression and the increase of HbF production

The comparison of the response to Hydroxyurea and Rapamycin were performed using the two-phase liquid culture (protocol A). The efficiency of Rapamycin in inducing both γ-globin mRNA and HbF approaches that found with HU as revealed in preliminary data shown in Figure 18, which in addiction indicates a fair relationship between increase of γ-globin mRNA and HbF.

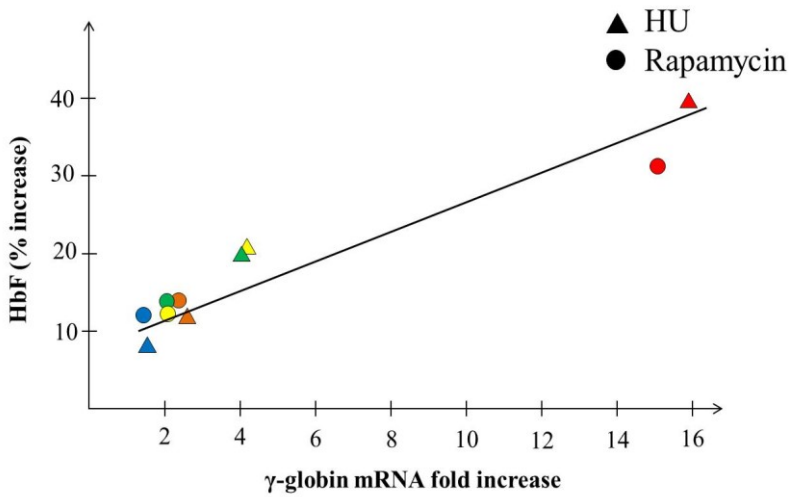


Figure 18. Relationship between increase of HbF and of γ-globin mRNA. The percentage of increase is calculated using the algorithm HbF increase = (% HbF induced cells - % HbF uninduced cells)/(100 - % HbF uninduced cells) x 100.fold increase). The induction of γ-globin mRNA is expressed as a fold increase.

1.3.4. Effects of Sirolimus on HbF induction: comparison with HU

A total of 39 treatments with Rapamycin at the final concentration of 500 nM were carried out. Figure 19 shows that ErPCs from different β-thalassemia patients increases the production of HbF at different level. Indeed, a first analysis of the *in vitro* response is suggested to identify patients to be selected for a possible clinical trial. The full set of data obtained are shown in Table 3.

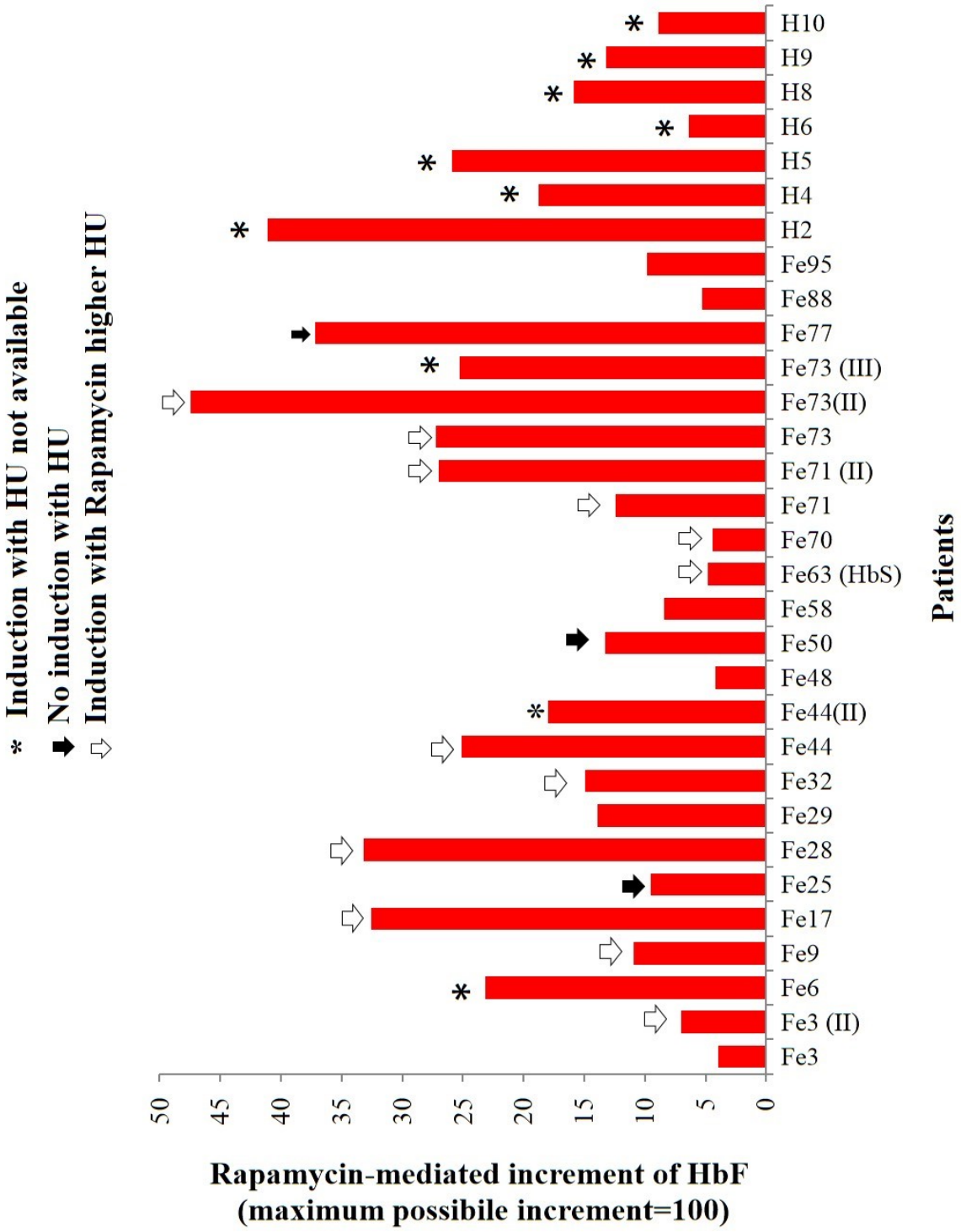


Figure 19. Induction of HbF in ErPCs treated with 500 nM Rapamycin (Sirolimus). HbF production in erythroid precursors cell was obtained by HPLC analysis of cells lysates.

Table 3. Induction of HbF in ErPCs treated with 500 nM Rapamycin (RAPA) or 100 µM Hydroxyurea (HU). The values HbF % indicate the percentage of fetal haemoglobin with respect to total haemoglobin in cell lysates.

Patients	HbF starting level	HbF (%) RAPA	HbF (%) HU	HbF %increase RAPA	HbF %increase HU	Genotype
Fe3	26.26	29.2	39.78	3.98	18.33	$\beta^0$39/β^+IVSI-110
Fe3 (II)	17.55	23.32	22.17	6.99	5.6	$\beta^0$39/β^+IVSI-110
Fe6	67.82	75.28	n.d.	23.18	n.d.	$\beta^0$39/$\beta^0$39
Fe9	45.26	51.24	49.08	10.92	6.98	$\beta^0$39/$\beta^0$39
Fe17	47.34	64.49	57.36	32.57	19.03	$\beta^0$6 (-A)/β^+IVSI-110
Fe24	32.47	33.52	27.01	1.55	-8.08	β^+IVSI-110/β^+IVSI-110
Fe25	27.47	34.35	25.33	9.48	-2.95	β^+IVSI-110/β^+IVSI-110
Fe28	70.93	79.91	73.96	33.17	13.52	$\beta^0$39/$\beta^0$44
Fe29	52.91	59.45	64.36	13.89	24.31	$\beta^0$39/$\beta^0$39
Fe32	48.30	56.01	52.62	14.91	8.36	$\beta^0$39/$\beta^0$39
Fe42	38.42	36.39	41.50	-3.29	5.00	$\beta^0$39/β^+IVSI-110
Fe43	6.63	7.31	10.17	0.73	3.79	β^+IVSI-6/β^+IVSI-110
Fe44	62.52	71.93	70.10	25.11	20.22	$\beta^0$39/$\beta^0$39
Fe44(II)	72.48	77.42		17.95		$\beta^0$39/$\beta^0$39
Fe48	31.32	34.22	34.06	4.22	3.99	β^0IVSI-1/β^+-101
Fe50	63.80	68.60	60.05	13.26	-10.36	$\beta^0$39/$\beta^0$39
Fe57	22.54	15.68	19.71	-8.86	-3.65	β^+IVSI-6/β^+IVSI-6
Fe57(II)	12.78	11.69	13.9	0	1.28	β^+IVSI-6/β^+IVSI-6
Fe58	37.85	43.06	42.01	8.38	6.70	β^+IVSI-110/β^+ -86
Fe63	1.22	5.99	10.32	4.8	9.21	HbS/$\beta^0$39
Fe70	53.84	55.89	54.9	4.4	2.29	$\beta^0$39/β^+IVSI-110
Fe71	33.25	41.55	40.82	12.43	11.34	$\beta^0$39/β^+IVSI-110
Fe71(II)	30.97	49.42	33.21	26.98	3.2	$\beta^0$39/β^+IVSI-110
Fe73	81.03	86.20	83.42	27.25	12.60	$\beta^0$39/$\beta^0$39
Fe73(II)	77.27	88.06	78.74	47.47	6.4	$\beta^0$39/$\beta^0$39
Fe73(III)	77.27	83.01		25.25		$\beta^0$39/$\beta^0$39
Fe77	89.88	93.64	89.84	37.15	0	$\beta^0$39/$\beta^0$39
Fe88	48.12	50.88	50.78	5.32	5.13	β^+IVSI-110/$\beta^0$39
Fe95	53.11	57.71	68.00	9.81	31.76	$\beta^0$39/β^+IVSI-110
H1	4.6	6.5		1.9		$\beta^0$39/β^+IVSI-110
H2	35.0	61.7		41.11		β^+-28/ β^+IVSI-110
H3	66.4	99.1		97.3		β^0IVS2-1/β^0IVS2-1
H4	3.4	21.6		18.8		β^+IVSI-110/β^+IVSI-110
H5	44.6	59.0		25.9		β^+IVSI-6/β^+IVSI-6
H6	20.9	26.0		6.4		β^+IVSI-110/β^+IVSI-110
H7	93.4	97.7		65.2		β^0IVS2-1/β^0IVS2-1
H8	15.4	28.9		15.9		β^+ -28/$\beta^0$44 (-C)
H9	34.5	43.1		13.2		$\beta^0$37(-T)/$\beta^0$44 (-C)
H10	7.8	16.0		8.9		β^+IVSI-6/β^+IVSI-6

A good correlation between induction by Sirolimus and starting HbF levels in β-thalassemia patients was found. This is shown in Figure 20, which indicates that in ErPCs the endogenous starting levels of HbF is variable. Interestingly, ErPCs from high HbF expressing patients are responsive to Sirolimus with higher efficiency with respect to ErPCs from low HbF expressing patients.

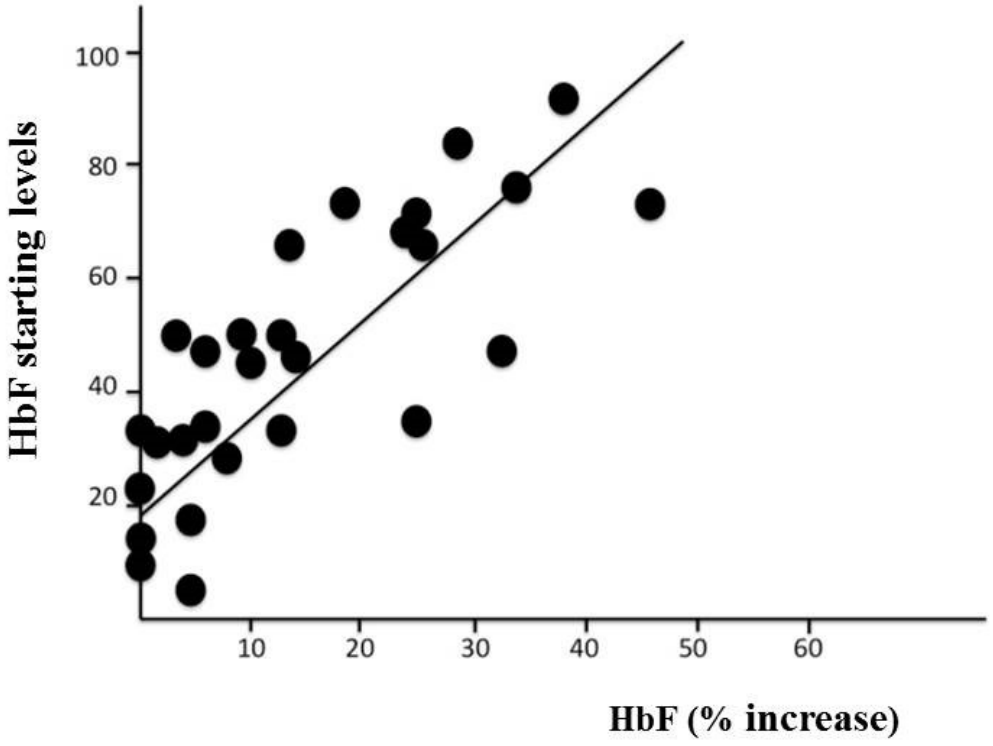


Figure 20. Relationship between % induced increase of HbF in Sirolimus-treated ErPCs and starting levels of HbF.

As far as the relationship between response to Sirolimus and response to Hydroxyurea, the data reported in Figure 21 show that different classes of ErPCs can be identified: (a) displaying no response, (b) preferentially induced by Sirolimus (c) preferentially induced by Hydroxyurea and induced by both Sirolimus and Hydroxyurea.

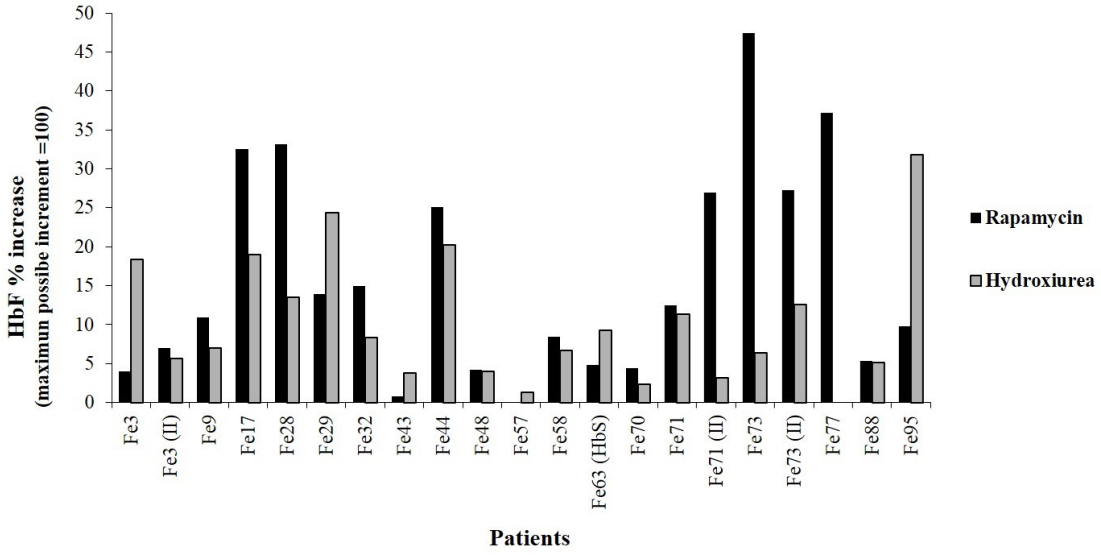


Figure 21. Increase of HbF production in ErPC from different β-thalassemia patients treated with Sirolimus (in black) or Hydroxyurea (in grey).

Figure 22 highlights that Rapamycin augments the production of fetal hemoglobin also in Hydroxyurea-resistant ErPCs at very high levels. For example, the percentage of increment in ErPCs of patient Fe73 treated with Rapamycin is 47.47%, while it is only 6.4 after the treatment with Hydroxyurea. These findings indicate that Rapamycin represents a concrete alternative to Hydroxyurea in not responder patients.

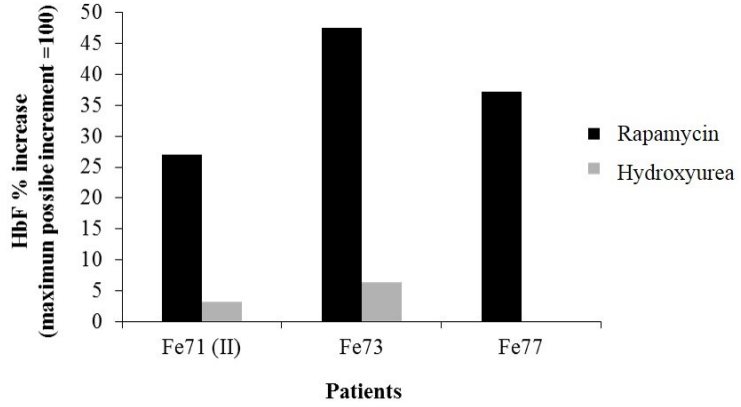


Figure 22. Increase of HbF production in Hydroxyurea-resistant ErPCs.

1.3.5. Microarray analysis of the transcriptome and induction of fetal hemoglobin

We have first verified whether the pattern of gene expression differentiates ErPCs from patients expressing high and low levels of HbF. This is indeed the case, as shown by the results displayed in Figure 23.

Gene expression analysis of RNAs from ErPCs producing high and low HbF was performed using Agilent Whole Human Genome Oligo Microarray platform (Agilent Technologies), following manufacturer's procedures. GeneSpring GX 11 software (Agilent Technologies) was used to analyze results. Data transformation was applied to set all the negative raw values to 1.0, followed by a quantile normalization. A filter on low gene expression was used to keep only the probes classified as Detected in at least one sample by the software. Array results were submitted in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). Genes were ordered according to fold-change, from the most down-regulated to the most up-regulated and the ordered gene list was analyzed. For pathway analysis we used GeneSpring GX v.11 and GeneGo software through the functions for finding significant pathways.

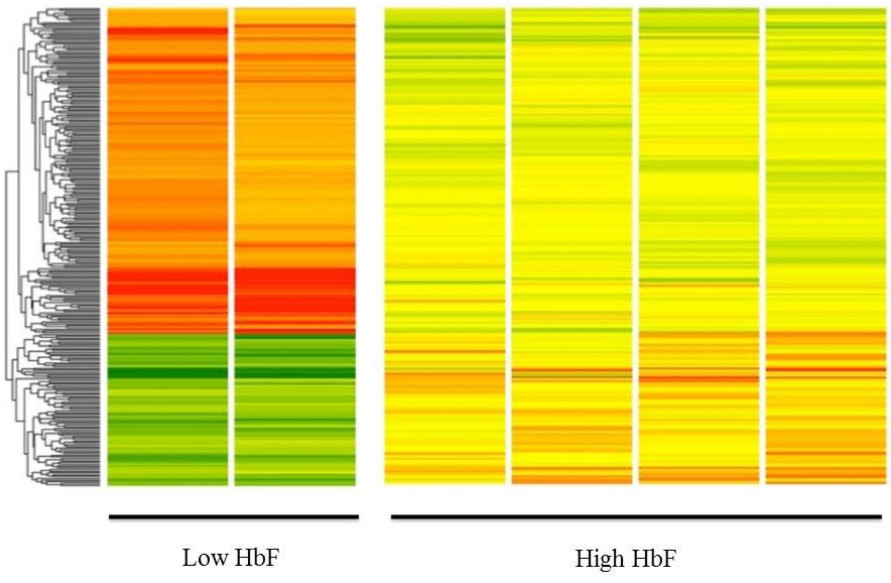


Figure 23. Differential transcriptomic profile in ErPCs from patients producing low and high levels of HbF.

The data obtained led to think that the gene expression would change after treatment of ErPCs with different inducers. This was clearly validated by the experiment shown in Figure 24 and demontrating a high level of gene expression change following treatment of human erythroleukemia K562 cells with Mithramycin.

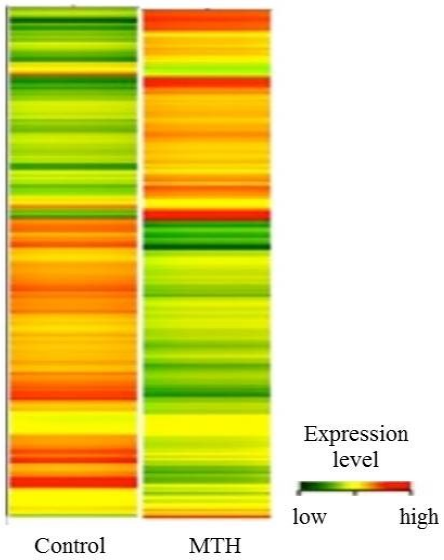


Figure 24. Differential transcriptomic profile in K562 untreated cells (control) and cells treated with the HbF inducer Mithramycin (MTH). A change in gene expression profile is evident after the treatment with the well-known inducer Mithramycin.

After these preliminary data showing that (a) the transcriptomic profile differentiate ErPC expressing different HbF values (Figure 23) and (b) the trascriptomic profile differentiate untreated human erythroleukemia K562 cells versus K562 cells treated with an HbF inducer (Figure 24), microarray analysis was performed to verify whether changes of gene expression occur between Sirolimus- and Hydroxyurea-treated ErPCs. The results obtained clearly demonstrate that the transcriptomic profile is different when untreated cells are compared with cells treated with Sirolimus, Hydroxyurea or Sirolimus plus Hydroxyurea (Figure 25). As inducer-specific signature was also confirmed by comparing up-regulated and down-regulated genes (limiting the study to the 200 most up-regulated and down-regulated), as shown in Table 4.

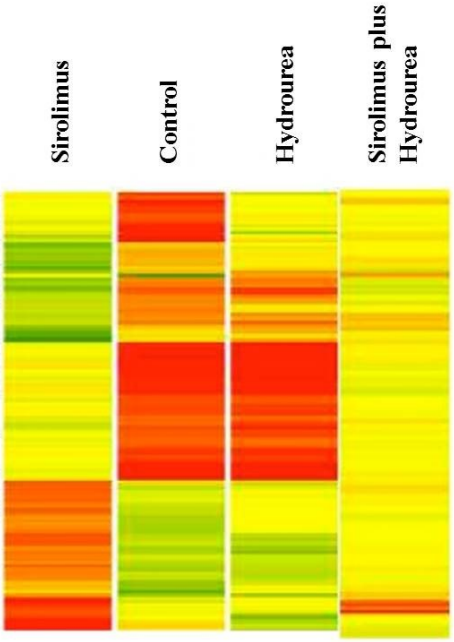


Figure 25. Differential transcriptomic profile in untreated ErPCs and cells treated with Sirolimus, Hydroxyurea or Sirolimus plus Hydroxyurea, as indicated. As clearly evident, the transcriptomic profile displays significative differences among the samples analysed.

Table 4. Data obtained from transcriptomic analysis of RNA isolated from ErPCs treated with Sirolimus, HU, or Sirolimus plus HU. This preliminary analysis was obtained limiting the study to the 200 most up-regulated and down-regulated genes.

Genes	Number	Comments/examples
Top up-regulated genes in SIROLIMUS-treated cells and up-regulated in HU-treated cells	110/200	This finding supports the concept of a different overall gene expression in SIROLIMUS- and HU-treated ErPCs
Top up-regulated genes in HU-treated cells and up-regulated in SIROLIMUS -treated cells	107/200	This finding supports the concept of a different overall gene expression in SIROLIMUS- and HU-treated ErPCs
Genes up-regulated only in SIROLIMUS -treated cells	90/200	35/90 were found up-regulated in ErPCs treated with both SIROLIMUS and HU
Genes up-regulated only in HU-treated cells	93/200	79/93 were found up-regulated in ErPCs treated with both SIROLIMUS and HU
Top down-regulated genes in SIROLIMUS-treated cells and down-regulated in HU-treated cells	103/200	This finding supports the concept of a different overall gene expression in SIROLIMUS- and HU-treated ErPCs
Top down-regulated genes in HU-treated cells and down-regulated in SIROLIMUS-treated cells	152/200	This finding supports the concept of a different overall gene expression in SIROLIMUS- and HU-treated ErPCs
Genes down-regulated only in SIROLIMUS-treated cells	97/200	95/97were found down-regulated in ErPCs treated with both SIROLIMUS and HU
Genes down-regulated only in HU-treated cells	48/200	44/48 were found down-regulated in ErPCs treated with both SIROLIMUS and HU

In agreement with the conclusion that HU and Rapamycin induce a differential effects on the overall gene expression, possibly reflecting the different mechanism of action, the response of ErPCs from a sub-set of β-thalassemia patients to the treatment with Sirolimus + HU were investigated. The results are shown in Figure 26 and 27. These data suggest that the combined treatments using Sirolimus plus HU is more efficient than HU in a subset of patients in inducing HbF.

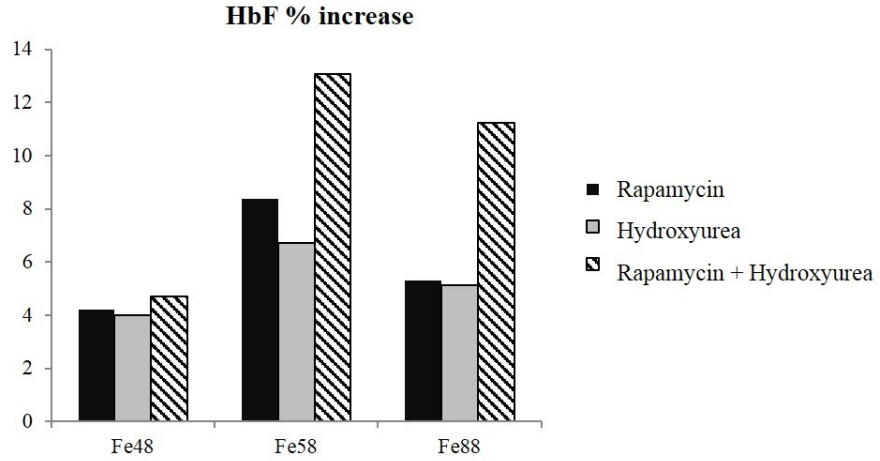


Figure 26. Effects of combined treatment of ErPC with Rapamycin plus Hydroxyurea in comparison with single treatments. The induction of fetal hemoglobin were higher performing the combined treatment with the two HbF inducers with respect to single treatments.

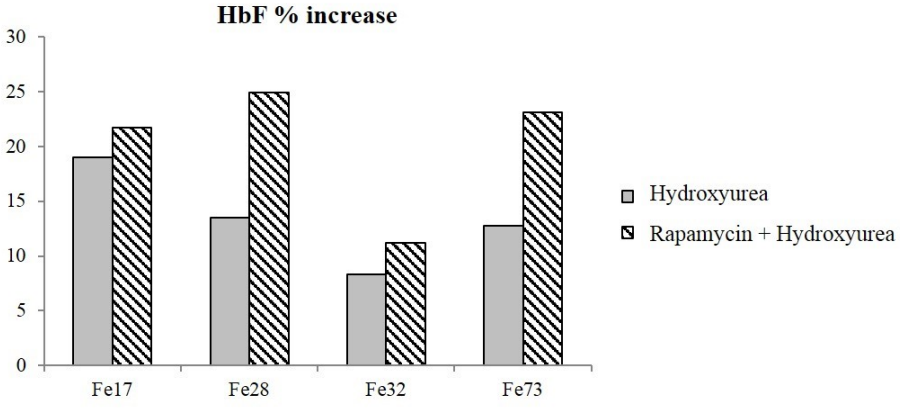


Figure 27. Effects of combined treatment of ErPC with Rapamycin plus Hydroxyurea in comparison with Hydroxyurea treatment. The induction of fetal hemoglobin were higher performing the combined treatment with the two HbF inducers with respect to Hydroxyurea treatment.

1.4. DISCUSSION

Sirolimus (as Sirolimus or Rapamune) was approved by the U.S. Food and Drug Administration for prevention of acute rejection in renal transplant recipients. Preliminary *in vitro* studies revealed that Rapamycin is able to induce preferentially γ-globin gene expression in human erythroleukemia cell line and erythroid precursors cells (ErPCs) from patients with β-thalassemia and sickle cell anemia. In ErPCs, the induction of γ-globin transcripts were correlated with the increase of production of fetal hemoglobin, but surprisingly not associated with cytotoxicity and cell growth inhibition. Moreover, the dosages that was found eff ective *in vitro* were very similar to those described to be present in the blood of kidney transplanted patients treated with Rapamycin (Gummert et al., 1999). The availability of new treatments for rare diseases may be offered by the use of drugs already approved for other indications, since pharmacokinetics, adsorption, route of administration, distribution and metabolism have been already established. For all these reasons, we have further investigated the potential of Rapamycin in the therapy of β-thalassemia and sickle cell anemia. Erythroid precursors cells from peripheral blood were isolated from 33 patients with 14 different genotypes. The *in vitro* induction of γ-globin gene expression was as expected heterogeneous as well as the induction of HbF. Interestingly, ErPCs from high HbF expressing patients were responsive to Sirolimus with higher efficiency with respect to ErPCs from low HbF expressing patients, demonstrating a relationship. A comparison of the responses of ErPCs to Hydroxyurea treatment and Rapamycin treatment revealed that different classes of ErPCs can be identified: displaying no response, preferentially induced by Sirolimus, preferentially induced by Hydroxyurea and induced by both Sirolimus and Hydroxyurea. This examination suggest that a first analysis of the *in vitro* response is of primary important to identify patients to be selected for a possible clinical trial. We then focused on the effects of the combined treatment of Rapamycin and Hydroxyurea. By checking the gene expression profile of ErPCs from patients expressing high and low levels of HbF, a differential pattern was found between the two classes of erythroid precursors cells. Nevertheless, the transcriptomic profile was different when untreated cells were compared with cells treated with Sirolimus, Hydroxyurea or Sirolimus plus Hydroxyurea. Hence, further analysis were carried on in order to examine the effect of the combined treatment in ErPCs. The data obtained revealed that the co-administration of Sirolimus plus HU were more efficient than HU in a subset of patients in inducing HbF. For all these findings, we believe that Rapamycin

should be taken into account for a clinical trial aimed at identify the best fetal hemoglobin inducer with the view to establish a personalized therapy of β-thalassemia and sickle cell anemia.

Recently, the European Commission has granted Orphan Drug Designation to Sirolimus (Rapamycin) for the treatment of β-thalassemia intermedia and major. The decision has been taken by the Commission on December 14, 2015, and follows the positive opinion issued by the Committee for Orphan Medicinal Products (COMP) of the European Medicines Agency (EMA) on 12 November 2015, recommending the designation of the medicinal product containing Sirolimus as an orphan medicinal product for the indication: treatment of β-thalassemia intermedia and major.

2. RESVERATROL

2.1. INTRODUCTION

2.1.1. Chemical structure and natural sources

Resveratrol is a natural compound that exists in both *cis* and *trans* isomeric forms. *Trans*-Resveratrol (Figure 28), also referred to as 3,5,4′-tri-hydroxystilbene, belongs to the stilbene class of polyphenolic compounds.

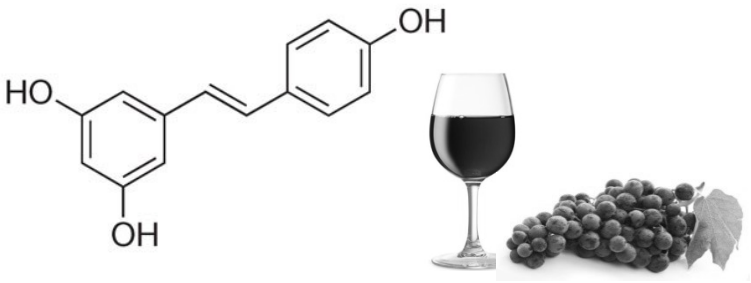


Figure 28. Chemical structure of *trans*-Resveratrol and natural sources.

Resveratrol was first isolated by Takaoka M. from the roots of the white hellebore (*Veratrum grandiflorum*) (Takaoka et al., 1940). In 1963, the compound was identified in the roots of *Polygonum cuspidatum*, a plant used in the traditional medicine Ko-jo-kon and described as a "prescription for inflammation, carcinogenesis, and cardiovascular diseases" in "Mei-I-Betsu-Roku," a book on materia medica published about 1500 years ago (Nonomura et al., 1963). Surprisingly, the actions described for Ko-jo-kon are almost identical to those recently described for Resveratrol. The first reported detection of *trans*-Resveratrol in *Vitis Vinifera* dates to 1976 (Langcake et al., 1976) and later in wine in 1992 (Siemann et al., 1992). Phytoalexins such as Resveratrol are not present in healthy vine leaves or berries, but are abundant in mature vine wood, and their synthesis is stimulated by exposure to ultraviolet (UV) rays (Langcake et al., 1977). *Botrytis cinerea* infection in grapes leads to the exclusive synthesis of Resveratrol in the leaf epidermis and grape skins. Since grape skins are not fermented during white wine production, only red wines contain noticeable amounts of Resveratrol. Resveratrol is also present in human diet i.e. in fruits

such as peanuts, strawberry, blueberry, cranberry, mulberry, lingberry, sparkleberry, bilberry, soy beans, and pomegranates. Table 5 indicates the concentration of Resveratrol in natural foods (Mukherjee et al., 2010).

Table 5. The amount of Resveratrol found in natural foods.

Source	Resveratrol concentration
100% Natural peanut butter	~0.65 µg/g
Bilberries	~16 ng/g
Blueberries	~32 ng/g
Boiled peanut	~5.1 µg/g
Cranberry raw juice	~0.2 mg/L
Dry grape skin	~24.06 µg/g
Grapes	0.16-3.54 µg/g
Peanut butter	0.3-1.4 µg/g
Peanuts	0.02-1.92 µg/g
Pistachios	0.09-1.67 µg/g
Ports and sherries	<0.1 mg/L
Red grape juice	~0.5 mg/L
Red wines	0.1-14.3 mg/L
Roasted peanuts	~0.055 µg/g
White grape juice	~0.05 mg/L
White wines	<0.1-2.1 mg/L

2.1.2. Biological activity

The "French paradox" is a term that was generated in 1992 based on epidemiological data from French people who had a low incidence of coronary heart diseases (CHD) despite the consumption of a diet in high saturated fat. This prompted researchers to think about a possible reason, and led Renaud and de Lorgeril (Renaud et al., 1992) to propose that moderate wine consumption (almost 57% of the overall alcoholic drink consumption in France) explained this apparent discrepancy, and further suggesting that a decrease in platelet aggregation may be the main factor of the effect on CHD. The potential Resveratrol benefits for health have led to many reports; Figure 29 summarizes the potential roles of Resveratrol in different processes.

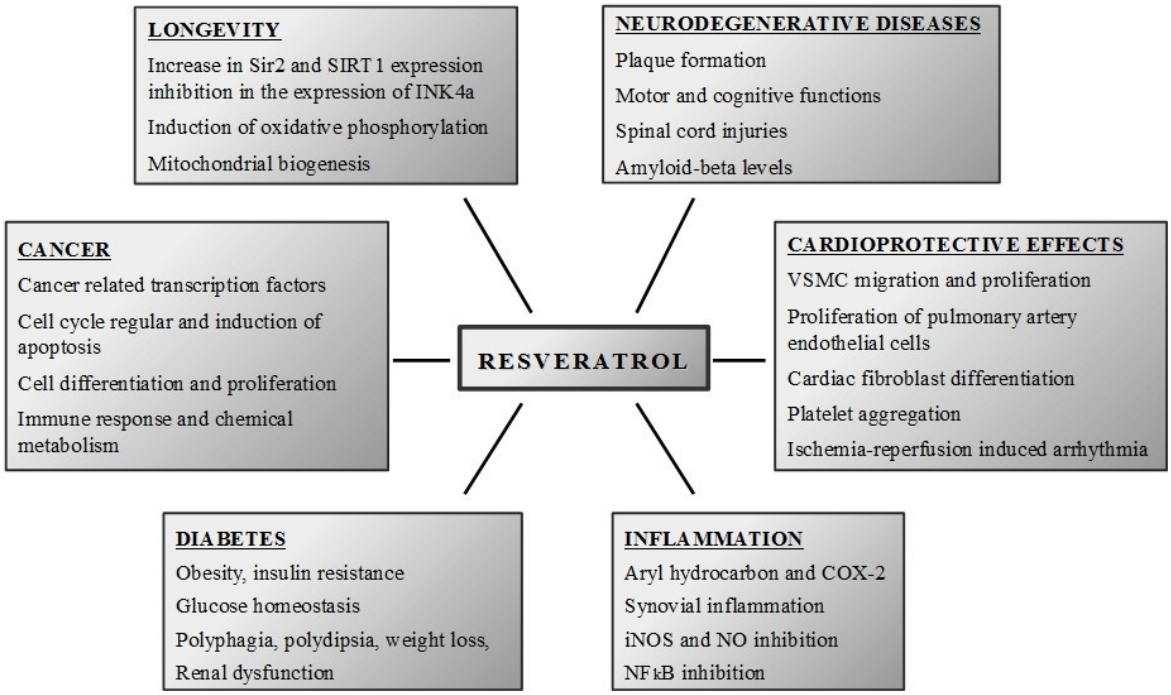


Figure 29. Examples of biological activities of Resveratrol.

The antioxidant activity of Resveratrol is demonstrated by various studies and it is reported to have anti-inflammatory, cardiovascular protective, and cancer chemopreventive properties. It was shown to target prostaglandin H_2 synthases since it is a potent inhibitor of both the cyclooxygenase and peroxidase reactions of COX-1. The chemopreventive effects have been demonstrated *in vivo* in a mouse skin cancer model (Jang et al., 1997). The ability of Resveratrol to directly induce apoptosis has been demonstrated in various tumor models, including promyelocytic leukemia (Surh et al., 1999), prostate cancer (Hsieh et al., 1999) and breast cancer (Mgbonyebi et al., 1998).

It has been demonstrated that Resveratrol activates the p53-dependent apoptotic process (Kai et al., 2010) in prostate cancer. The antiproliferative effect of Resveratrol might results from the influence on the expression levels of phosphorylation status of cell cycle regulators such as cyclin E, cyclin A, cdc2, and Rb, thereby arresting cell division cycle at S/G2 phase transition (Ragione et al., 1998). Moreover, the anticancer property of Resveratrol is also due to their ability to inhibit ribonucleotide reductase in mammalian cells, as Hydroxyurea (Fontecave et al., 1998). So far no adverse effects of Resveratrol in humans has been reported and bioavailability studies report that it is well absorbed and rapidly metabolized, mainly into sulfo and glucuronides conjugates which are eliminated in

urine (Cottart et al., 2010). Interestingly, Resveratrol exhibited minimal toxicity toward normal hematopoietic cells (Clèment et al., 1998).

2.1.3. Preliminary studies on erythroid differentiation

In 2001, Resveratrol was found to induce differentiation of K562 cells and to augment fetal hemoglobin (HbF) production in K562 cells and in erythroid precursors isolated from SCA patients (Rodrigue et al., 2001). The interest on Resveratrol in the therapy of β-thalassemia is also supported by its antioxidant activity; in fact, oxidative stress has an important role in β-hemoglobinopaties. It has been showed that RBC, as well as platelets and polymorphonuclear leukocytes (PMN) derived from such patients, are under oxidative stress (Amer et al., 2006). They contain lower levels of reduced glutathione (GSH), the major antioxidative compound, concomitant with increased levels of reactive oxygen species (ROS) compared with that of their normal counterparts. Oxidative stress in thalassemia is caused primarily by degradation of unstable Hb, which results in free globin chains and heme. Another contributing factor is iron overload due to increased intestinal absorption and regular blood transfusions (Amer et al., 2008). Oxidative stress in blood cells affects their function: RBC precursors undergo apoptosis during their development in the bone marrow, resulting in ineffective erythropoiesis and as a consequence, a severe chronic anemia.

The molecular mechanisms of growth arrest and differentiation induced by Resveratrol in K562 cells have been described: the induction of γ-globin synthesis is linked to impairment of cell proliferation, increased $p21^{cip1}$ expression and inhibition of cdk2 activity. Resveratrol up-regulates the transcription factor Erg1 by an Erk1/2-dependent mechanism and the presence of an Erg1 consensus sequence in the $p21^{cip1}$ promoter indicates that this transcription factor directly regulates the expression of the cdk inhibitor (Ragione et al., 2003).

In 2012 Fibach et al. further investigated the activity of Resveratrol in K562 cell line and erythroid precursors cells from β-thalassemia patients and healthy donors. A dose-response inhibition of spontaneous and H_2O_2-induced levels of ROS was clearly detectable after 1 hour of treatment of K562 cells and the inhibition of growth was associated with the erythroid differentiation of this cell line. In erythroid precursors cells isolated from β-thalassemia patients, Resveratrol inhibited both spontaneous and H_2O_2-induced levels of

ROS, increased the content of GSH and reduced lipid peroxides. Moreover, it increased fetal hemoglobin as well as γ-globin mRNA significantly and reproducibly in cultures of all the patients studied (Figure 30) (Fibach et al., 2012).

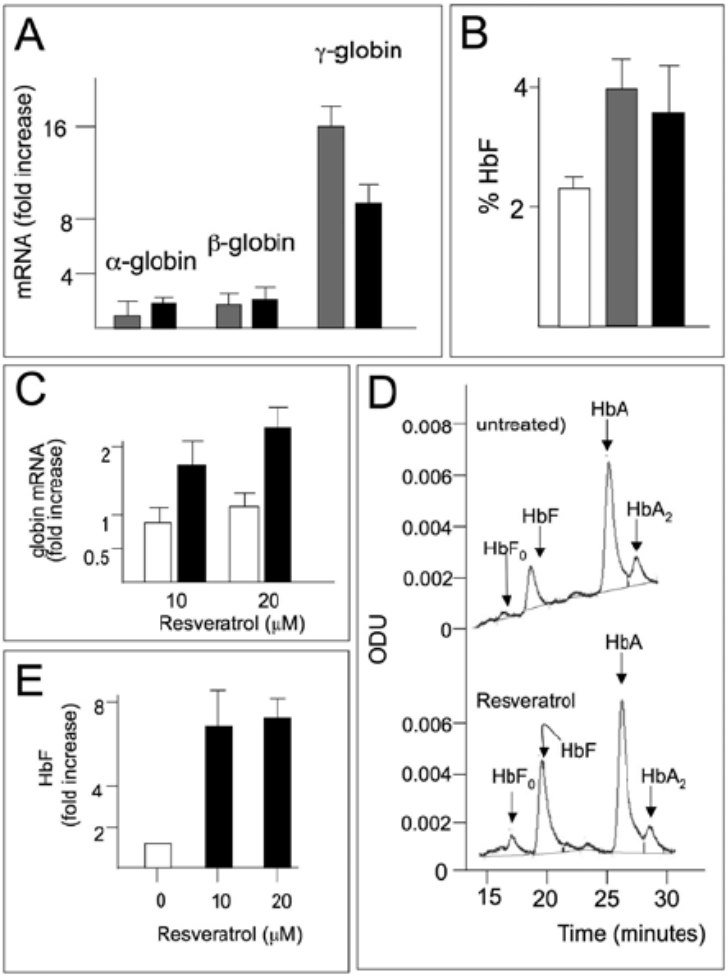


Figure 30. Effects of resveratrol on γ-globin mRNA and HbF in normal and thalassemia erythroid precursors. Erythroid precursors derived from the peripheral the peripheral blood of normal donors (**A** and **B**) or thalassemia patients (**C**-**E**). Effects on γ-globin mRNA accumulation (fold increase with respect to control untreated cells) (**A** and **C**) in normal (**A**) and β-thalassemia cultures (**B**). Resveratrol (black boxes) was used at 20 μM and the HbF inducer Mithramycin (grey boxes, 100 nM) was used as a positive control. Effects on HbF production in normal (**B**) and thalassemia cultures (**D** and **E**). Representative HPLC analyses of lysates from control cultures (upper part of panel) and resveratrol-treated cultures (100 μM, lower part of panel) from a β-thalassemia patient are shown in **D**. (From Fibach et al., 2012)

2.1.4. *In vitro* effectiveness of trans-Resveratrol nutraceuticals

Fourteen brands of Resveratrol-containing nutraceuticals (PC01-PC14) were evaluated in order to verify their actual Resveratrol content by the research group of Prof. Gambari, in collaboration with Prof. Sacchetti (Life Sciences and Biotechnology Department, University of Ferrara). Products included pure Resveratrol capsules or multi-ingredient formulations with standardized amounts of Resveratrol and other phytochemicals. Samples were analyzed for total *trans*-Resveratrol, and only five out of 14 brands, including PC02 (Transmax® Biotivia), had near label values, compliant with Good Manufacturing Practices (GMP) requirements (95–105% content of active constituent) The antiproliferative and the effects on erythroid differentiation of K562 cells were carried out with all these fourteen Resveratrol-containing nutriaceuticals. PC02 exhibited a greater induction of differentiation towards benzidine-treated human leukemic K562 cells when compared to pure Resveratrol. The induction of globin transcripts in K562 cell line treated with PC02 was thereafter evaluated (Table 6).

Table 6. Fold induction of γ-globin mRNA in K562 cells treated with PC02 at different concentrations or *trans*-Resveratrol. The levels of expression of γ-globin mRNA were analyzed by RT-qPCR.

Treatment	Concentration of Resveratrol	fold γ-globin
PC02 20 μg/mL	83,4 μM	2,23
PC02 10 μg/mL	41,7 μM	2,36
PC02 5 μg/mL	20,9 μM	2,08
Resveratrol	40 μM	2,16

Treatments with PC02 were performed also in erythroid precursors cells from a β-thalassemia patient. The effects on globin genes expression and fetal hemoglobin production are reported in Tables 7 and 8.

Table 7. Fold induction of globin mRNAs in erythroid precursors cells isolated from β-thalassemia patient Fe9. The levels of expression of globin mRNAs were analyzed by RT-qPCR.

	Treatment	fold α-globin mRNA	fold β-globin mRNA	fold γ-globin mRNA
Fe9	PC02 10 µg/mL	0,89	0,90	2,31
	Resveratrol	0,70	0,80	3,56

Table 8. HPLC analysis of hemoglobins in erythroid precursors cells from Fe9.

	Hemoglobin	% Hb
Fe 9	HbF	10,32
Untreated	α_4	17,71
	HbA_0	6,20
	HbA_2	62,40
Fe9	HbF	18,84
PC02 10 µg/mL	α_4	15,87
	HbA_0	4,66
	HbA_2	60,63
Fe9	HbF	24,46
Resveratrol 40 µM	α_4	10,37
	HbA_0	7,83
	HbA_2	57,32

2.2. MATERIALS AND METHODS

2.2.1. Research protocol

The study was carried out at the Pediatric Oncohematology Unit, Santa Chiara Hospital (Pisa) and led by Dott. Claudio Favre in collaboration with Prof. Roberto Gambari, University of Ferrara.

Aim: evaluation of the antioxidant activity of the Resveratrol-nutriaceutical Transmax® 500 Biotivia in β-thalassemia patients, after a single daily administration for 12 weeks.

Patients recruitment:

Inclusion criteria

- patients with β-thalassemia, diagnosed by clinical and hematological criteria;
- over the age of 18 years;
- Hb lower than 10 g/dL;
- HIV-negative and HCV-negative;
- preference for splenectomized patients.

Exclusion criteria

- pregnant or lactating women;
- kidney disease, heart, liver, respiratory, neurological, endocrine, or other blood disease;
- inclusion in other clinical research protocol;

Number of patients: 6.

Study phases:

1. Screening of the patients
2. Single daily administration of Transmax® (90 days)
3. Follow-up (30 days)

Laboratory tests:

During treatment

A. Blood sampling before the transfusion. Culture of erythroid precursors cells isolated from 20-30 mL of peripheral blood. HPLC analysis and RT-qPCR analysis after the two-phase liquid culture of the cells. Evaluation of oxidative stress parameters: ROS, GSH, lipid peroxidation.

B. Every 15 days: general analysis (blood count, blood urea nitrogen, creatinine, electrolytes, transaminases, alkaline phosphatase, gamma-GT, total bilirubin and fractional urine, reticulocytes) with the aim of evaluating the possible side effects and eventually to stop treatment.

During follow-up

At day 120: blood sampling before the transfusion and culture of erythroid precursors cells. HPLC analysis and RT-qPCR analysis after the two-phase liquid culture of the cells. Evaluation of oxidative stress parameters: ROS, GSH, lipid peroxidation. General analysis (blood count, blood urea nitrogen, creatinine, electrolytes, transaminases, alkaline phosphatase, gamma-GT, total bilirubin and fractional urine, reticulocytes); evaluation of hematological parameters, soluble transferrin receptor, erythropoietin, HbF.

Potential adverse effects:

No toxic or adverse effects of Resveratrol in humans have been described until now. A recent study that evaluated the safety of oral Resveratrol in ten subjects by administering a single dose up to 5 grams reported no serious adverse effects.

Additional studies:

The 20-30 mL of blood drawn just before the usual transfusion will be used for the culture of erythroid precursors. After the two-phase liquid culture, cell lysates will be analyzed by HPLC and RT-qPCR will be performed in order to evaluate the level of expression of globin transcripts.

2.2.2. The nutraceutical product

Transmax® Bioivia actually contains 500 mg of Polygonum Cuspidatum extract per capsule consisting of 99% of trans-Resveratrol (495 mg per capsule of the trans-Resveratrol). The company that makes Transmax® declare that the product contains only bio-enhanced pharmaceutical-grade Resveratrol, obtained by advanced and patented processes. Analytical tests carried out by the company Biotivia provide the contents of a single pills reported in Table 9.

Table 9. Contents of a unit of Transmax® Biotivia as declared by the company.

	mg/capsule
Unit content weight	531.00
trans-polydatin	0.69
cis-polydatin	< 0.10
Polydatin glicoside	0.41
trans-Resveratrol	511.00
cis-Resveratrol	< 0.001
Free Resveratrol	511.00
Total Resveratrol	511.40

2.2.3. Culture of erythroid precursors isolated from peripheral blood of β-thalassemia patients

The culture of human erythroid progenitors were performed as described on page 32 of this PhD thesis.

2.2.4. Real Time quantitative PCR analysis

For gene expression analysis, RNA was extracted and quantified as described on pages 33 and 34 of this PhD thesis. 500 ng of total RNA were reverse transcribed using random hexamers (see on page 34). Quantitative real-time PCR assay of γ-globin, β-globin

and α-globin mRNAs was carried out using gene-specific double fluorescence labelled probes in the iQ™5 Multicolor Real-Time PCR Detection System (BIORAD, Hercules, California, USA). The fluorescent reporter and the quencher were: 6-carboxyfluorescein (FAM) and 6-carboxy-N,N,N',N'-tetramethylrhodamine (TAMRA), respectively. The nucleotide sequences are reported in Table 10.For quantification, the reference gene was human glyceraldehyde phosphate dehydrogenase (GAPDH); this probe was fluorescence-labelled with VIC (Applied Biosystems, Monza, Italy). Fold increase was determined by comparing the "threshold" cycle (C_T) value relative to the amplification of the cDNA to be quantified with that of the amplified reference GAPDH sequence (ΔC_T value). In order to quantify the increase of the levels of expression of a target gene in different PCR products, the differences between ΔC_T values were considered ($\Delta\Delta C_T$). The $\Delta\Delta C_T$ represents the negative exponent in the equation $2^{-\Delta\Delta CT}$, quantifying the fold increase of expression of the target gene in several samples, taking as unchanged the levels of GAPDH expression.

Table 10. Sequences of primers and probes used in quantitative PCR reactions.

PRIMER/PROBE	SEQUENCE
primer forward α-globin	5'-CACGCGCACAAGCTTCG-3'
primer reverse α-globin	5'-AGGGTCACCAGCAGGCAGT-3'
sonda α-globin	5'-FAM-TGGACCCGGTCAACTTCAAGCTCCT-TAMRA-3'
primer forward β-globin	5'-CAAGAAAGTGCTCGGTGCCT-3'
primer reverse β-globin	5'-GCAAAGGTGCCCTTGAGGT -3'
sonda β-globin	5'-FAM-TAGTGATGGCCTGGCTCACCTGGA-TAMRA-3'
primer forward γ-globin	5'-TGGCAAGAAGGTGCTGACTTC-3'
primer reverse γ-globin	5'-TCACTCAGCTGGGCAAAGC-3'
sonda γ-globin	5'-FAM-TGGGAGATGCCATAAAGCACCTGC-TAMRA-3'

RT-qPCR assay was carried out also following the multiplex method and as a reference gene RPL13A (60S Ribosomal protein L13a). The protocol is described on page 35 of this PhD thesis.

2.2.5. High performance liquid chromatography (HPLC) analysis

Human erythroid precursor cells were harvested, counted (ZF Coulter Counter, Coulter Electronics, Hialeah, FL, USA), washed once with PBS, and pellets were lysed in water by freeze and thaw cycles and recovered by centrifugation at 14,000g for 30 min and 4 °C. Hb proteins present in the lysates were separated by cation-exchange HPLC, using Synchropak CM300 (250x4.6 mm, Synchron Inc, Lafayette IN)

2.3. RESULTS

2.3.1. Patients characterization

Patients were recruited following all the ethical requirements and the approval of the Ethical Committees of Santa Chiara Hospital, Pisa. Blood samples were collected from β-thalassemia patients after signature of the informed consent form. In this PhD thesis four patients will be taken into consideration. Table 11 reports the mutations identified in the β-globin gene for each patient.

Table 11. Genetic detail of the patients studied.

Th1	Unknown genotype
Th6	β^{+}IVSII-74; 5'UTR +20 (C>T)
Th13	β^{+}IVSII-74/ β^{+} -87
Th19	β°39/ β°39

During Resveratrol administration, blood sample have been collected every 20 days. One sample has been taken during the follow-up. The zero-time culture of erythroid precursors cells has been treated with Resveratrol in order to evaluate the in *vitro* response. Figure 31 provides a scheme of the performed analysis. Moreover, blood samples have been collected at different time points after one year.

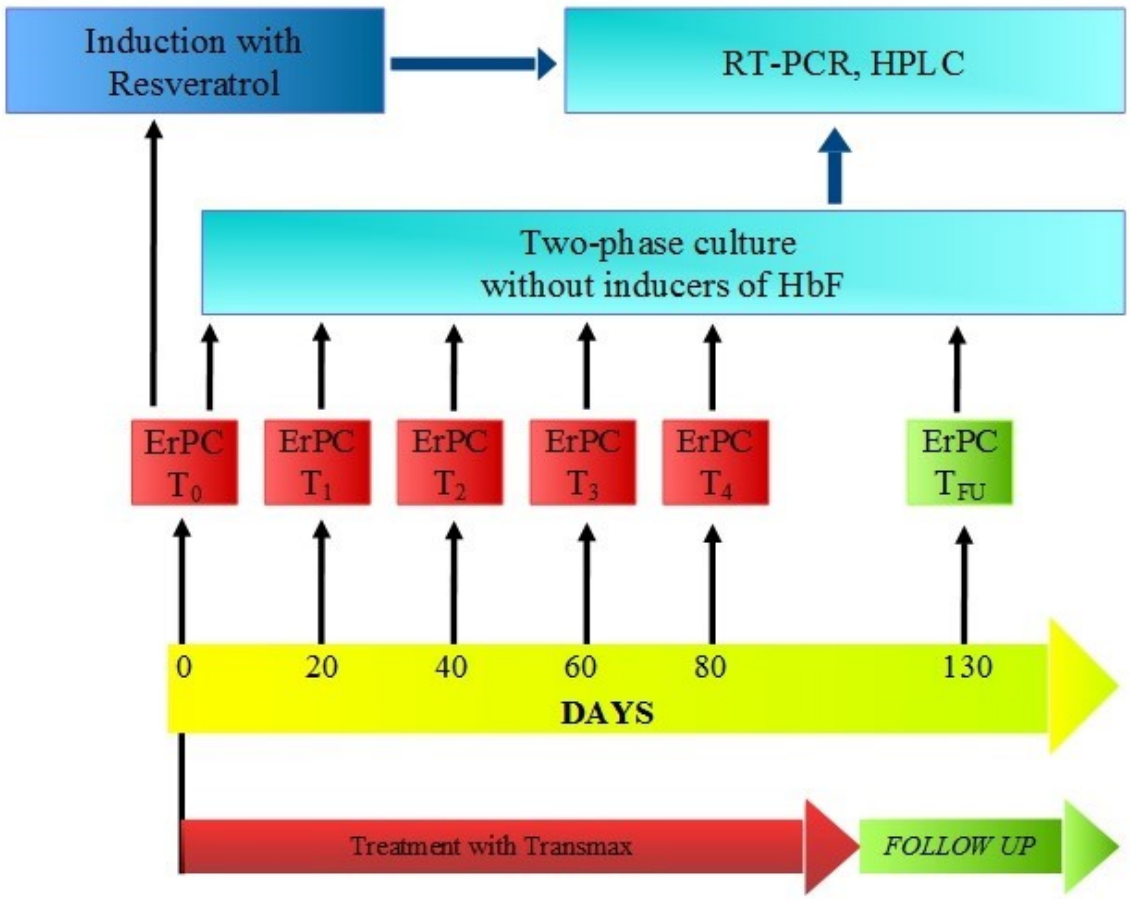


Figure 31. Overview of the analysis performed during the administration of Transmax® in β-thalassemia patients. Blood samples have been collected every 20 days during Resveratrol administration, but also during the follow-up. RT-PCR and HPLC analysis have been performed after the two-phase liquid culture of the human erythroid precursors cells.

2.3.2. Analysis of γ-globin gene expression during *in vivo* administration of Resveratrol

Resveratrol induction of γ-globin mRNA is evident in the four β-thalassemia patients. In fact, the ratio between γ-globin and α-globin fold induction remains at substantial levels under *in vivo* administration of Transmax®. Figures 32, 33, 34 and 35 report also the relative expression of γ-globin in relation to α-globin fold mRNA during period when patients have not taken Resveratrol-nutraceutical.

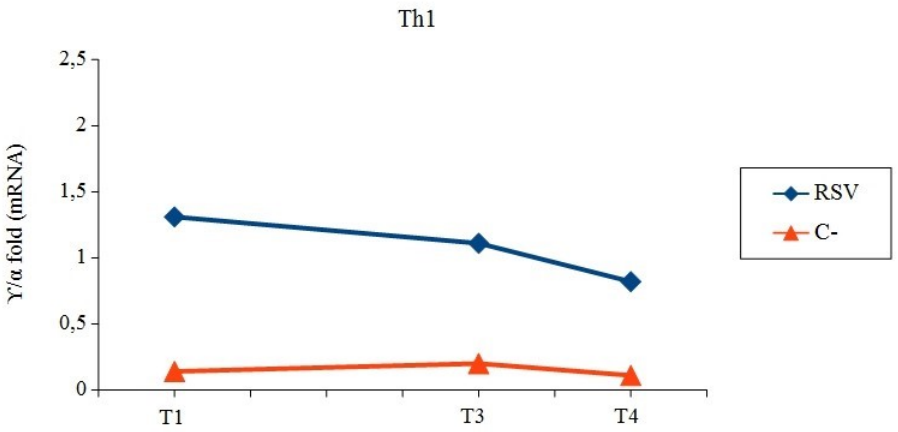


Figure 32. Expression of γ-globin mRNA under *in vivo* administration of Resveratrol (blue curve) and one year after stopping treatment (red curve) in patient Th1. The ratio between γ-globin and α-globin fold induction remains at substantial levels during *in vivo* administration of Transmax®.

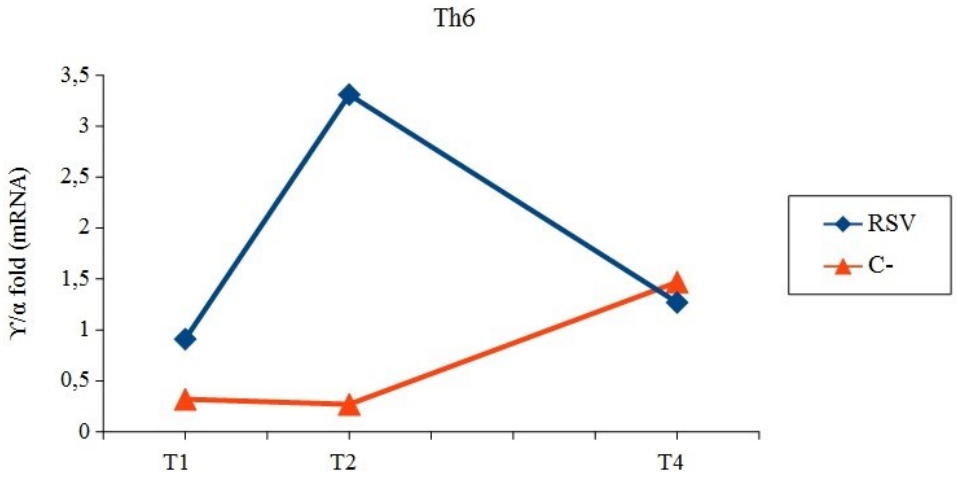


Figure 33. Expression of γ-globin mRNA during *in vivo* administration of Resveratrol (blue curve) and one year after stopping treatment (red curve) in patient Th6. The ratio between γ-globin and α-globin fold induction under Resveratrol administration tend to be higher than during treatment-free period.

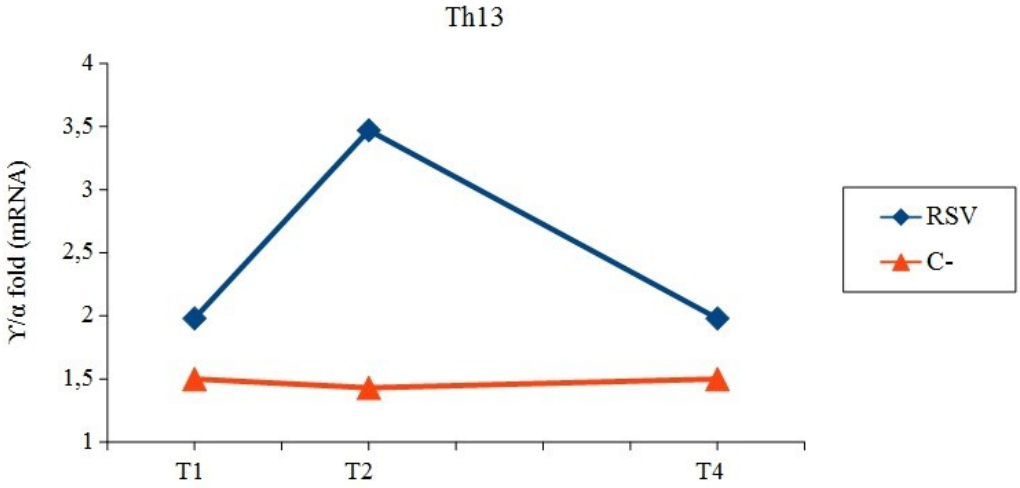


Figure 34. Expression of γ-globin mRNA during *in vivo* administration of Resveratrol (blue curve) and one year after stopping treatment (red curve) in patient Th13. The ratio between γ-globin and α-globin fold induction remains at substantial levels during *in vivo* administration of Transmax®.

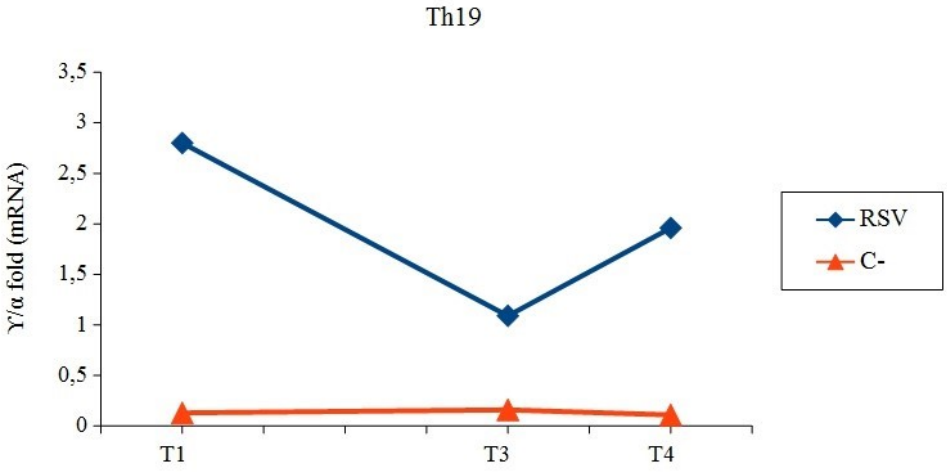


Figure 35. Expression of γ-globin mRNA during the *in vivo* administration of Resveratrol (blue curve) and one year after stopping treatment (red curve) in patient Th19. The ratio between γ-globin and α-globin fold induction under *in vivo* administration of Transmax® is generally higher than during treatment-free period.

2.3.3. HPLC analysis of the fetal hemoglobin production of erythroid precursors cells during Resveratrol administration

The production of fetal hemoglobin of erythroid precursors cells isolated from peripheral blood of patients Th1, Th6 and Th19 has been assessed by HPLC analysis. An increase of production of HbF has been detected under Resveratrol administration, but also during follow-up. Figures 36, 37 and 38 represent the trend in HbF production.

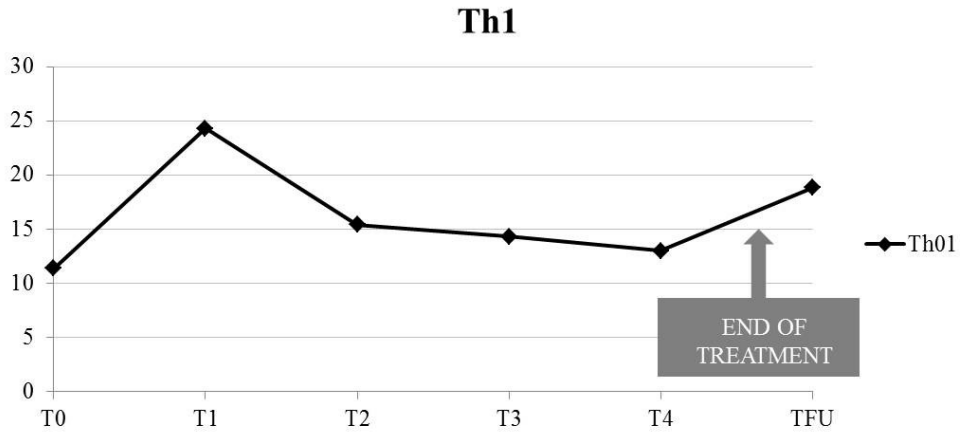


Figure 36. HPLC analysis of HbF production of ErPCs under Resveratrol administration in patient Th1. The maximum of fetal hemoglobin production in patients Th1 is obtained at T_1 (20 days of treatment).

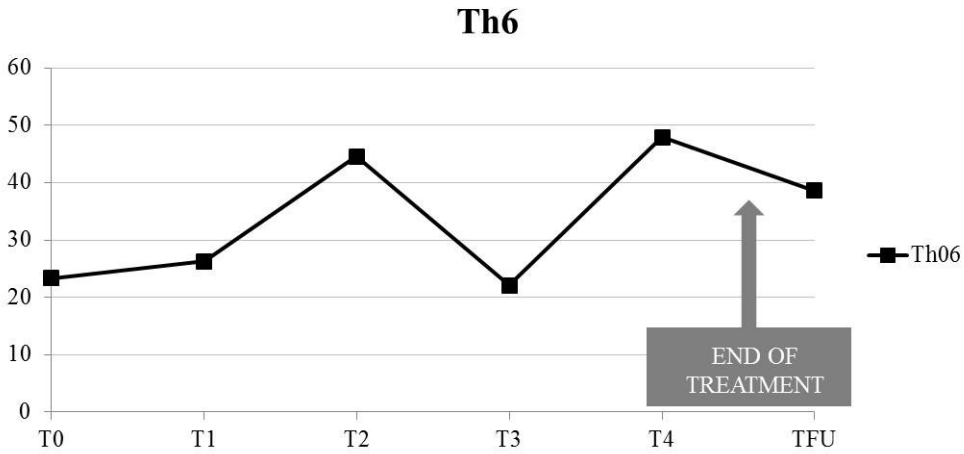


Figure 37. HPLC analysis of HbF production of ErPCs under Resveratrol administration in patient Th6. Fetal hemoglobin production in patients Th6 reaches a remarkable level, especially at T_2 (40 days of treatment) and T_4 (80 days of treatment).

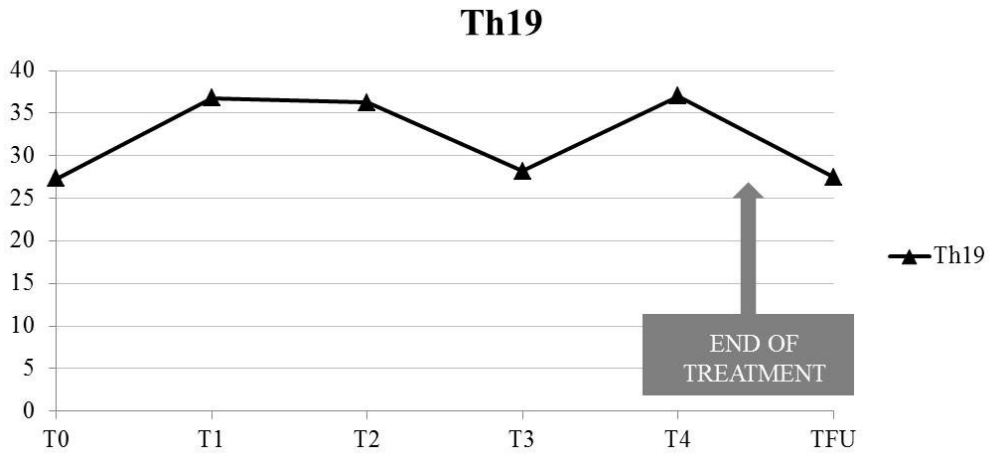


Figure 38. HPLC analysis of HbF production of ErPCs under Resveratrol administration in patient Th19. Fetal hemoglobin production in patients Th19 start from a high level. Nevertheless, it reaches considerable increase after 20, 40 and 60 days of treatment with Resveratrol.

The maximum of fetal hemoglobin production in patients Th1 is obtained at T_1 (20 days of treatment), when the percentage of increase is 14.61%. During follow-up, HbF production remains at higher level than before the treatment. Fetal hemoglobin production in patients Th6 reaches a remarkable level, especially at T_2 (40 days of treatment) and T_4 (80 days of treatment) when the percentage of increase is 27.64 and 32.02 respectively. During follow-up, the percentage of increase of HbF is still 19.86% with respect to T_0. Fetal hemoglobin production in patient Th19 start from a high level (27.31%). Nevertheless, after 20, 40 and 80 days of treatment a similar increase is observed (about 13%). During follow-up, the content of HbF in ErPCs are 27.49%, remaining at higher level than T_0.

The results obtained indicate that Resveratrol administration in β-thalassemia patients promotes the production of fetal hemoglobin in erythroid precursors cells with log-lasting effects. Since no relevant adverse effects of Resveratrol in humans have been reported, this phytoalexin might be proposed in the therapy of β-thalassemia and sickle cell anemia.

2.3.4. *In vitro* response to Resveratrol of erythroid precursors cells isolated from thalassemia patients is predictive of *in vivo* effects.

γ-globin gene expression has been evaluated by RT-PCR analysis. After the two-phase liquid culture, the zero-time erythroid precursors cells have been treated *in vitro* with Resveratrol 40 μM. γ-globin fold induction in Resveratrol treated cells has been obtained by comparison to untreated ErPCs. The level of expression of γ-globin during the administration of Transmax® has been compared to those of T_0. As depicted in Figure 39, there is a good correlation between the *in vitro* induction of γ-globin mRNA and the maximum level of expression of γ-globin trancript observed during the *in vivo* administration of Transmax®.

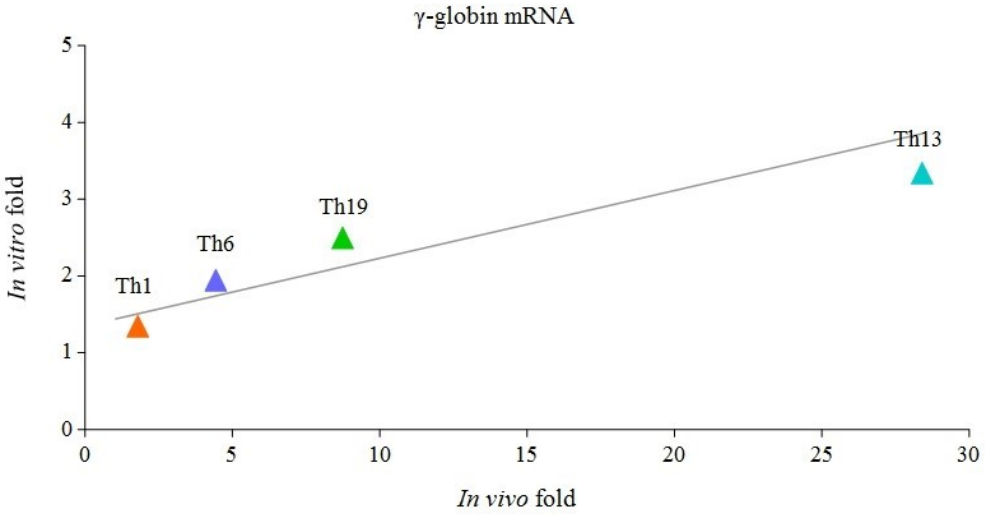


Figure 39. Relationship between the *in vitro* fold induction of γ-globin mRNA in ErPCs from β-thalassemia patients and the expression of γ-globin transcripts during the *in vivo* administration of Resveratrol.

2.4. DISCUSSION

The interest on Resveratrol has started since in 2001 this phytoalexin was found to induce differentiation of K562 cells and to augment fetal hemoglobin (HbF) production in K562 cells and in erythroid precursors isolated from SCA patients.

In this study, we have evaluate γ-globin gene expression and fetal hemoglobin production in erythroid precursors cells isolated from four β-thalassemia patients undergoing treatment with a Resveratrol-nutraceutical (Transmax®, Biotivia). In preliminary studies, Transmax® exhibited the greater *in vitro* induction of γ-globin transcripts and of fetal hemoglobin in ErPCs from a patients with β-thalassemia.

Resveratrol has been administered to patients at a concentration equal to that suggested in normal subjects, who take the nutraceutical product for its antioxidant activity. The study was carried out at the Pediatric Oncohematology Unit, Santa Chiara Hospital (Pisa) in collaboration with Dott. Claudio Favre. Patients were recruited following all the ethical requirements and the approval of the Ethical Committees of the Hospital. The treatment protocol has provided precise criteria for inclusion (β-thalassemia, diagnosis based on clinical, haematological criteria, more than 18 years, Hb less than 10 g / dL, negative for HIV and HCV, preferably splenectomized) and exclusion (pregnant or lactating women, kidney disease, heart, liver, respiratory, neurological, endocrine, or other hematologic disease in progress, the inclusion in other protocols for experimental drugs). Patients did not suspend blood transfusion. To check the effectiveness of the treatment, erythroid precursors cells were isolated from peripheral blood and cultured according to the two-phase liquid culture method. ErPCs isolated from the patient on starting day of treatment were treated with the commercial product Transmax® used in the trial. In order to evaluate the effects of the *in vivo* administration of Resveratrol, blood samples have been collected every 20 days under the treatment and also during follow-up. A first positive observation emerged from the clinical trial is that no adverse side effects was reported in the patients.

Four β-thalassemia patients have been taken into consideration in this PhD thesis. Resveratrol induction of γ-globin mRNA was evident during the treatment of the four β-thalassemia patients. The relative expression of γ-globin in relation to α-globin fold mRNA after one year, when patients have not taken Resveratrol-nutraceutical, was found to be significantly lower. Moreover, the production of fetal hemoglobin analyzed by HPLC was increased during the treatment but also during follow-up. Finally, a good correlation between the *in vitro* induction of γ-globin mRNA in ErPCs at T_0 and the maximum level of expression of γ-globin transcripts during the *in vivo* administration of Transmax® has been found. This evidence suggest that *in vivo* cultures of erythroid precursors cells from β-thalassemic patients should be considered predictive of *in vivo* therapeutic effects. Taken together, these results suggest that the *in vivo* administration of Resveratrol-nutraceutical

might be repurposed into a wider number of patients with β-thalassemia but also sickle cell anemia, performing an extended follow-up. These experiments will be associated with more detailed clinical analysis and with further assessment of the production of fetal hemoglobin in reticulocytes.

3. PSORALENS

3.1. INTRODUCTION

3.1.1. Chemical description and characteristics

Coumarins, compounds belonging to the family of benzopyrones, can be distinguished in four main sub-types: the simple coumarins (e.g., coumarin, 7-hydroxycoumarin), the furanocoumarins, derived from the condensation of the coumarin nucleus with a five-membered furan ring (e.g., psoralen, angelicin), the pyranocoumarins, that consist of a six-membered ring attached to the coumarin nucleus (e.g., seselin, xanthyletin) and the coumarins substituted in the pyrone ring (e.g., warfarin) (Keating et al., 1997). Furocoumarins occur naturally in a number of vegetables, fruits and in cold pressed oils from citrus fruits, exhibiting a planar tricyclic structure, or an angular arrangement of the rings.

Psoralens (Figure 40A), planar compounds first isolated from *Psoralea corylifolia* (Jois et al., 1933), have been used in psoralens-UVA treatment (PUVA) for psoriasis, vitiligo (Honigsmann, 2001; McNeely et al., 1998), and mycosis fungoides (T-cell lymphoma) (McGinnis et al., 2003). The ability of these molecules to cause cell damage is due to covalent binding with DNA after UV irradiation; in fact, the planar tricyclic structure of psoralens has two photoreactive sites (3,4-pyrone and 4',5'-furan double bonds). The first step of the mechanism of action consists in the drug intercalation between DNA nucleotide bases, then the drug absorption of a UVA photon permits the covalent binding between the furan ring double bond and a pyrimidine residue (preferentially a thymine) of the DNA molecule; by absorption of a second UVA photon, a covalent binding between the lactone ring double bond and another pyrimidine base on the opposite strand of DNA results in a psoralen diadduct to DNA (da Silva et al., 2009). For this property, psoralens have also been utilized for the treatment of human lymphoma and of autoimmune diseases through the extracorporeal photochemotherapy (ECP) (Balogh et al., 1998; Plumas et al., 2003). Furthermore, psoralens have been described as anticancer (Wang et al., 2011; Panno et al., 2014), neuroprotective (Lee et al. 2005), antifungal and antibacterial agents (Céspedes et al., 2006).

The angular furanocoumarins Angelicin (ANG, Figure 40B) occurs in the fruit or root of *Angelica Archangelica* (Apiacee). This plant has been used in traditional and folk

medicine as a remedy against nervous headaches, fever, skin rashes, wounds, rheumatism, and toothaches (Bhat et al., 2011). In recent studies, stomachic, antiseptic, digestive, carminative, antitumor proprieties of this plant have been also reported (Biglar et al., 2014; Sigurdsson et al., 2005).

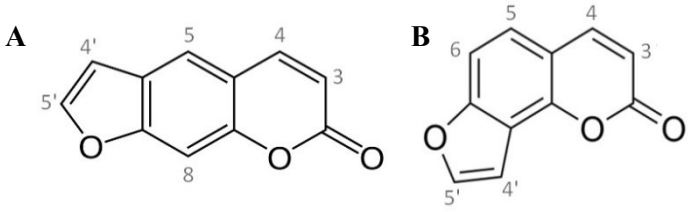


Figure 40. Chemical structures of linear (psoralen, A) and angular (angelicin, B) furanocoumarins. Furanocoumarins derive from the condensation of the coumarin nucleus with a five-membered furan ring.

3.1.2. Preliminary studies on erythroid differentiation

Aegle marmelos (Fam.Rutaceae) is an Indian medicinal plant whose fruits, stem and roots have been used in ethnomedicine for a variety of purposes, such as astringent, antidiarrhoeal, antidysentery, antipyretic, anti-vomiting, antiscourbutic, haemostatic, aphrodisiac and as an antidote to snake venom (Kirtikar et al., 1935; Nandkarni et al., 1979). As well as many studies pointed out the activity of the plant against such diabetes mellitus, fever, inflammation, gastric ulcer, cardiovascular diseases, Lampronti et al. observed the ability to significantly inhibit the *in vitro* proliferation of the human leukemic K562 cell line. Three compounds identified by GC-MS analysis of *Aegle marmelos C.* extracts, butyl p-tolyl sulfide, 6-methyl-4-chromanone and 5-methoxypsolaren (5-MOP) were able to inhibit cell proliferation together with the induction of erythroid differentiation of K562 cells (Lampronti et al., 2003).

Another powerful inducer of K562 cells is Angelicin; it has been demonstrated that this compound can increase the content of fetal hemoglobin in erythroid progenitors from normal subjects and the amount of γ-globin transcript in erythroid precursor cells isolated from β-thalassemia patients (Lampronti et al., 2003). The induction of erythroid differentiation of K562 cells and human erythroid progenitors was also achieved

combining the treatment of psoralens derivatives and ultraviolet light, at different concentrations and doses; the linear derivative 5-MOP, but especially the angular derivatives Angelicin and 4,6,4' -trimethylangelicin (TMA) were able to promote at very low concentrations a preferential increase of γ-globin mRNA (Viola et al., 2008).

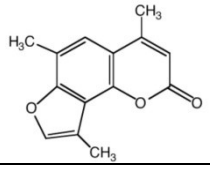
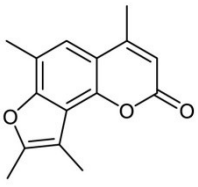
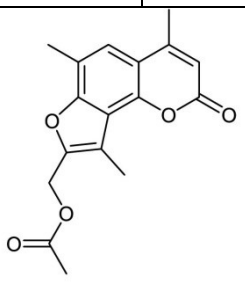
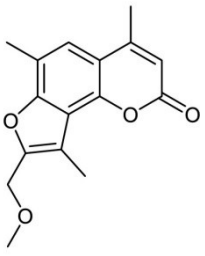
3.2. MATERIALS AND METHODS

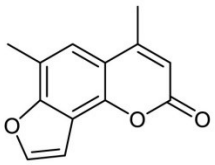
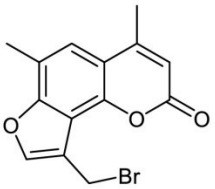
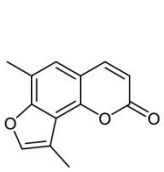
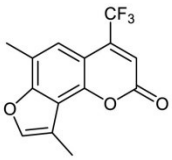
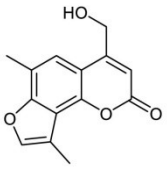
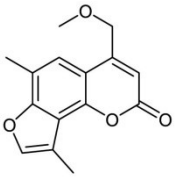
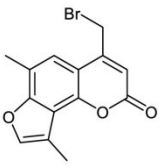
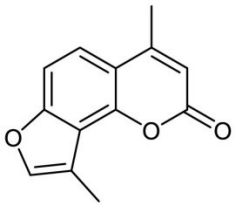
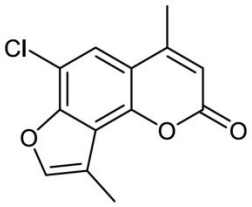
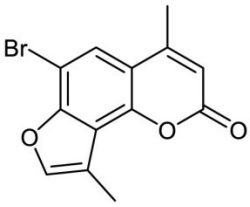
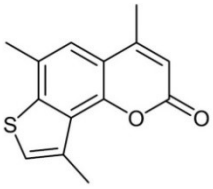
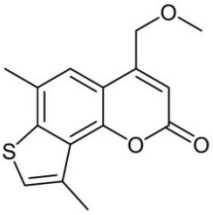
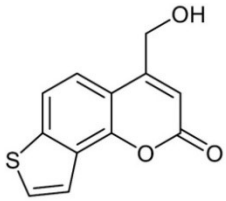
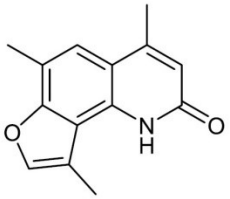
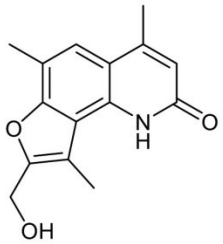
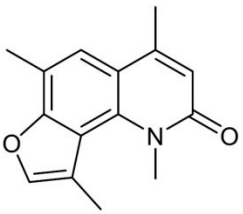
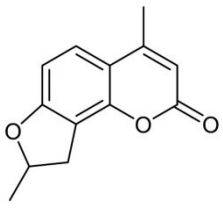
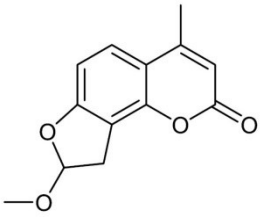
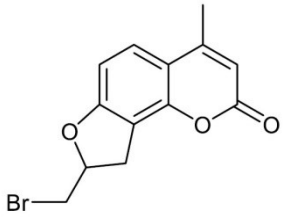
3.2.1. Development of new Angelicin derivatives

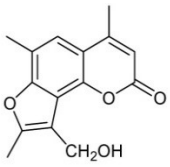
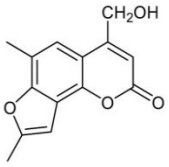
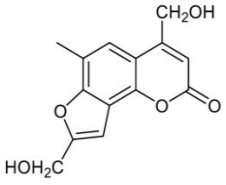
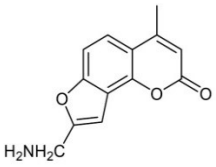
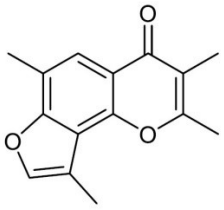
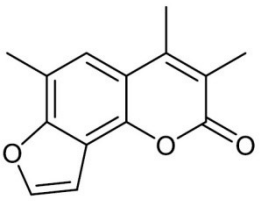
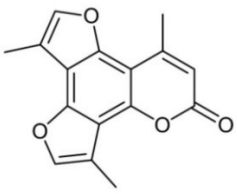
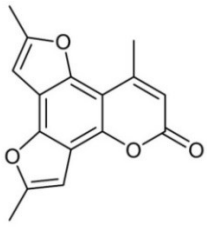
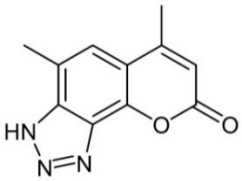
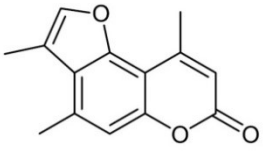
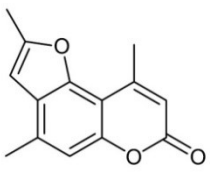
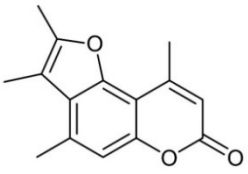
Despite the excellent results obtained, new analogues of Angelicin, in particular iso-psoralens, have been synthetized in order to reduce undesirable side effects, especially long-term ones, such as genotoxicity. Angular psoralens are indeed monofunctional isopsoralens isomers and cannot create inter-strand cross-link with the DNA. It is for this reason that 6-methylangelicins are potential photochemotherapeutic agents for the treatment of psoriasis (Guiotto et al., 1984).

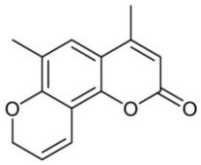
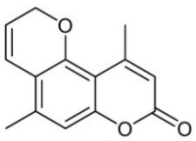
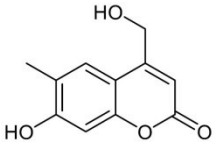
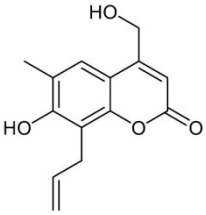
Prof. Adriana Chilin (Department of Pharmaceutical and Pharmacological Sciences of Padua, Italy), synthesized 38 Angelicin derivatives, called GMB (Table 12), that we tested in our research group.

Table 12. Structures of the new psoralen-like analogues.

SERIES	COMPOUNDS		
TMA		4,6,4' –trimethylangelicin	
4,6,4'	 **GMB-01** MW 242.27 Da	 **GMB-02** MW 300.31 Da	 **GMB-03** MW 272.30 Da

4,6	 **GMB-04** MW 214.22 Da		 **GMB-05** MW 307.14 D		
6,4'	 **GMB-06** MW 214.22 Da	 **GMB-07** MW 282.21 Da	 **GMB-08** MW 244.24 Da	 **GMB-09** MW 258.27 Da	 **GMB-10** MW 307.14 Da
4,4'	 **GMB-11** MW 214.22 Da	 **GMB-12** MW 248.66 Da	 **GMB-13** MW 293.11 Da		
Thio	 **GMB-14** MW 243.21 Da	 **GMB-15** MW 274.33 Da	 **GMB-16** MW 232.26 Da		
Furoquinolinones FQ	 **GMB-17** MW 227.26 Da	 **GMB-18** MW 257.28 Da	 **GMB-19** MW 241.29 Da		
Dihidro	 **GMB-20** MW 216.23 Da	 **GMB-21** MW 232.23 Da	 **GMB-22** MW 295.13 Da		

Others	 **GMB-23** MW 258.27 Da  **GMB-24** MW 244.24 Da  **GMB-25** MW 260.24 Da  **GMB-26** MW 229.23 Da
Chromon	 **GMB-27** MW 242.27 Da
3,4	 **GMB-28** MW 228.24 Da
Difuro	 **GMB-29** MW 268.26 Da  **GMB-30** MW 268.26 Da
Triazole	 **GMB-31** MW 215.21 Da
Allo	 **GMB-32** MW 228.24 Da  **GMB-33** MW 228.24 Da  **GMB-34** MW 242.27 Da

Pyran	 **GMB-35** MW 228.24 Da	 **GMB-36** MW 228.24 Da
Coumarin	 **GMB-37** MW 206.19 Da	 **GMB-38** MW 246.26 Da

3.2.2. Culture of human erythroleukemia cells K562 and erythroid precursors cells

The culture of human erythroleukemia cell line K562 and erythroid progenitors isolated from peripheral blood were performed as described on pages 31 and 32 of this PhD thesis. Cell growth analysis and benzidine test were carried out as described on page 33.

3.2.3. Real Time quantitative PCR analysis

For gene expression analysis, RNA was extracted and quantified as described on page 33 of this PhD thesis. 500 ng of total RNA were reverse transcribed using random hexamers (see on page 34). RT-qPCR assay was carried out also following the multiplex method and as a reference gene RPL13A (60S Ribosomal protein L13a). The protocol is described on page 35 of this PhD thesis.

3.2.4. High performance liquid chromatography (HPLC) analysis

Human erythroid precursor cells were harvested, counted (ZF Coulter Counter, Coulter Electronics, Hialeah, FL, USA), washed once with PBS, and pellets were lysed in water by freeze and thaw cycles and recovered by centrifugation at 14,000g for 30 min and 4 °C. Hb proteins present in the lysates were separated by cation-exchange HPLC, using Synchropak CM300 (250x4.6 mm, Synchron Inc, Lafayette IN)

3.3. RESULTS

3.3.1. Effects of GMB derivatives on cell growth and differentiation of K562 cell line

The screening of the compound sinthesized by the research group of Prof. Chillin started from the anti-proliferative and erythroid differentiation assay on K562 cell lines. For this purpose, cells were seeded at 20.000 cells/mL in plates and scalar doses of each compound were added. Three days after the tratment (72 hours), in the top phase of growth, cells were counted with Z2 Coulter Counter (Z™ Series BECKMAN COULTER®) instrument; the resulting IC50 values are listed in Table 13.
The benzidine-colorimetric test to evaluate the erythroid differentiation of K562 cells was performed on 5th, 6th, 7th day of culture. In Table 13 is shown the maximum level of differentiation obtained from each compound.

Table 13. IC50 values and maximum level of differentiation of K562 cells after the treatment with Angelicin analogues (GMBs). It is evident that several compounds are able to induce the erythroid differentiation of the human erythroleukemia cell line K562, but only four of them induced the differentiation of more than 15% of cells.

Series	Compound	IC50	Maximum level of differentiation
4,6,4'	GMB-01	427,3 μM	12% (800 μM)
	GMB-02	> 800 μM	3% (800 μM)
	GMB-03	447,4 μM	5% (800 μM)
4,6	GMB-04	> 800 μM	3% (50 μM)
	GMB-05	76,8 μM	5% (200 μM)
6,4'	GMB-06	77,5 μM	20% (800 μM)

	GMB-07	> 800 μM	2% (50 μM)
	GMB-08	26,1 μM	62% (50 μM)
	GMB-09	≥ 800 μM	6% (800 μM)
	GMB-10	< 200 nM	0
4,4'	**GMB-11**	134,4 μM	3% (200 μM)
	GMB-12	37,4 μM	4% (100 μM)
	GMB-13	≥ 800 μM	1% (800 μM)
thio	**GMB-14**	763,4 μM	0
	GMB-15	> 800 μM	3% (800 μM)
	GMB-16	744,2 μM	2% (800 μM)
FQ	**GMB-17**	33,1 μM	6% (50 μM)
	GMB-18	413,9 μM	3% (200 μM)
	GMB-19	24,6 μM	11% (50 μM)
dihydro	**GMB-20**	79,8 μM	15% (200 μM)
	GMB-21	82,31 μM	35% (200 μM)
	GMB-22	34,2 μM	1% (50 μM)
other	**GMB-23**	40,3 μM	8% (800 μM)
	GMB-24	36,7 μM	4% (800 μM)
	GMB-25	> 800 μM	0
	GMB-26	23,4 μM	20% (50 μM)
chromons	**GMB-27**	> 800 μM	3% (800 μM)
3,4	**GMB-28**	> 800 μM	2% (800 μM)
difuro	**GMB-29**	> 800 μM	2% (800 μM)
	GMB-30	> 800 μM	1% (50 μM)
triazole	**GMB-31**	175 μM	12% (800 μM)
allo	**GMB-32**	> 800 μM	2% (50 μM)
	GMB-33	> 800 μM	4% (800 μM)
	GMB-34	> 800 μM	3% (800 μM)
pyran	**GMB-35**	124,1 μM	2% (200 μM)
	GMB-36	≥ 800 μM	3% (800 μM)
coumarin	**GMB-37**	343.8 μM	3% (50 μM)
	GMB-38	370.1 μM	2% (50 μM)

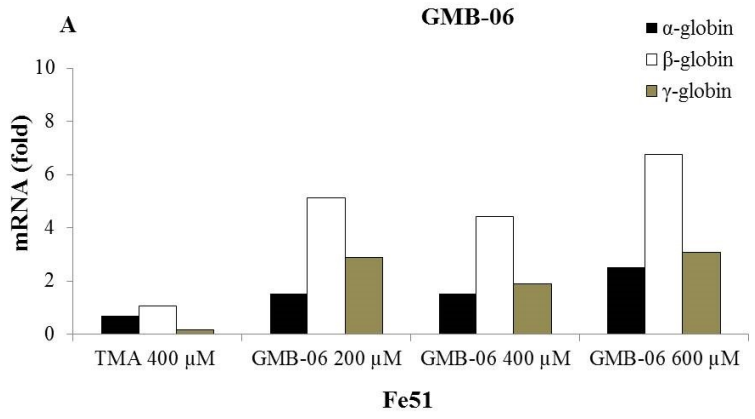
Considering the data obtained, the analogues underlined in Table 13 were taken into account for further analysis on globin gene expression on erythroid precursors cells from β-thalassemic patients. This four analogues, namely GMB-06, GMB-08, GMB-21 and GMB-26 were indeed the most effective molecules to benzidine test.

3.3.2. Effects of *in vitro* treatments with GMB derivatives on erythroid precursors cells

In order to verify if GMB analogues were able to induce HbF production, they have been tested in erythroid precursors cells derived from peripheral blood of β-thalassemic patients. Cells were cultured in the two-phase liquid culture system, as explained in "Matherial an Methods" and treated for five days with different concentration of the selected compound. Treatments have been tested over the range 200 μM to 600 μM and compared to the already known inducer trimethylangelicin (TMA) at the final concentration of 400 μM. IC50 values of the compounds on this erythroid cells were found at 14 mM for GMB-06, 2.1 mM for GMB-08, 1.76mM for GMB-21 and 7.66 mM for GMB-26. After RNA isolation, the results on the induction of globin genes expression were carried out through RT-qPCR analysis. HbF content of the developing erythroid cells were measured by HPLC analysis of lysates.

GMB-06

The first analogue was tested in erythroid precursor cells from patient Fe51 (β^+ IVS I-6 homozygous). As shown in Figure 41, GMB-06 600 μM produced a 3.07 fold increase of γ- globin mRNA, althought the compound was active at all concentration tested. However, HPLC analysis of cells lysates pointed out a maximum of 3.61% increase of HbF content in GMB-06 treated cells.



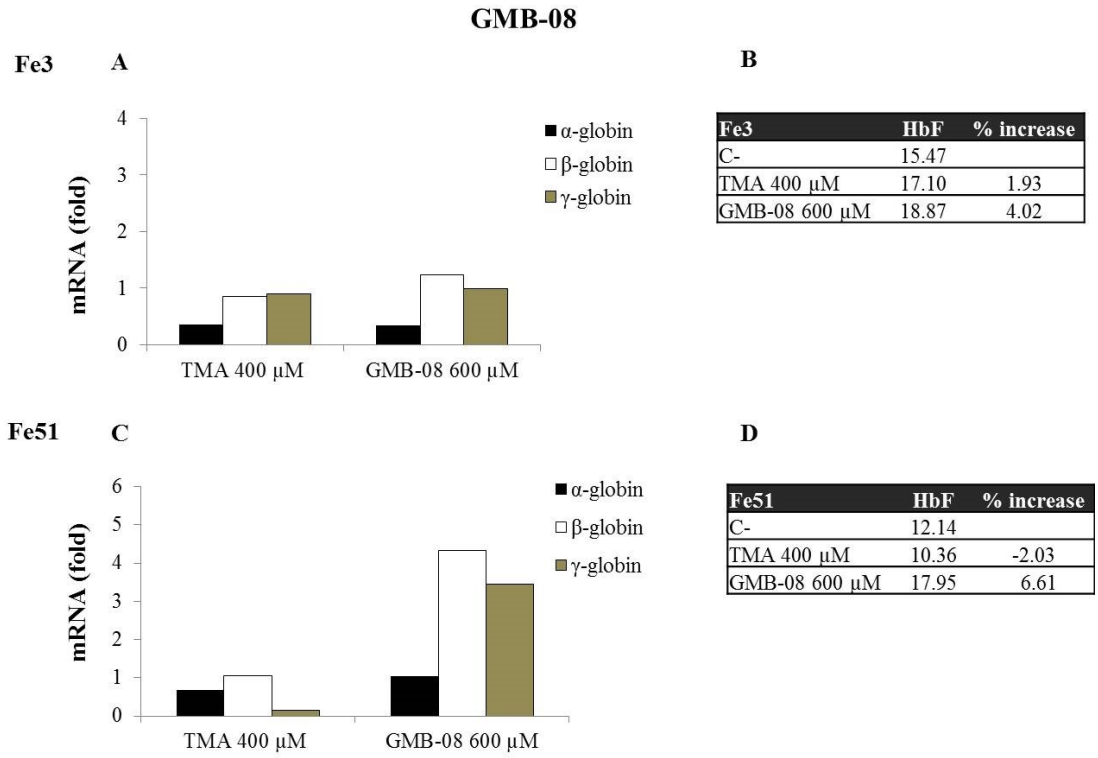
B

Fe51	HbF	% increase
C-	12.14	
TMA 400 μM	10.36	-2.03
GMB-06 200 μM	14.16	2.30
GMB-06 400 μM	15.31	3.61
GMB-06 600 μM	14.80	3.03

Figure 41. Effects of GMB-06 on the globin genes expression (A) and on HbF production (B) in erythroid precursors cells from patient Fe51. The highest accumulation of γ-globin genes expression was produced by GMB-06 at 200 μM and 600 μM (**A**). GMB-06 incresed HbF production, more effectively than TMA (**B**).

GMB-08

The compound was tested in two patients, Fe3 ($\beta^0$39/β^+IVS I-110) and Fe51 (β+ IVS I-6 homozygous) with positive results in both samples at 600 µM, since the percentage of increase of HbF content is considerable with respect to TMA. The data depicted in Figure 42 demonstrate that, in erythroid precursors cells isolated from Fe51, GMB-08 are able to induce the expression of γ-globin transcripts together with the production of HbF.



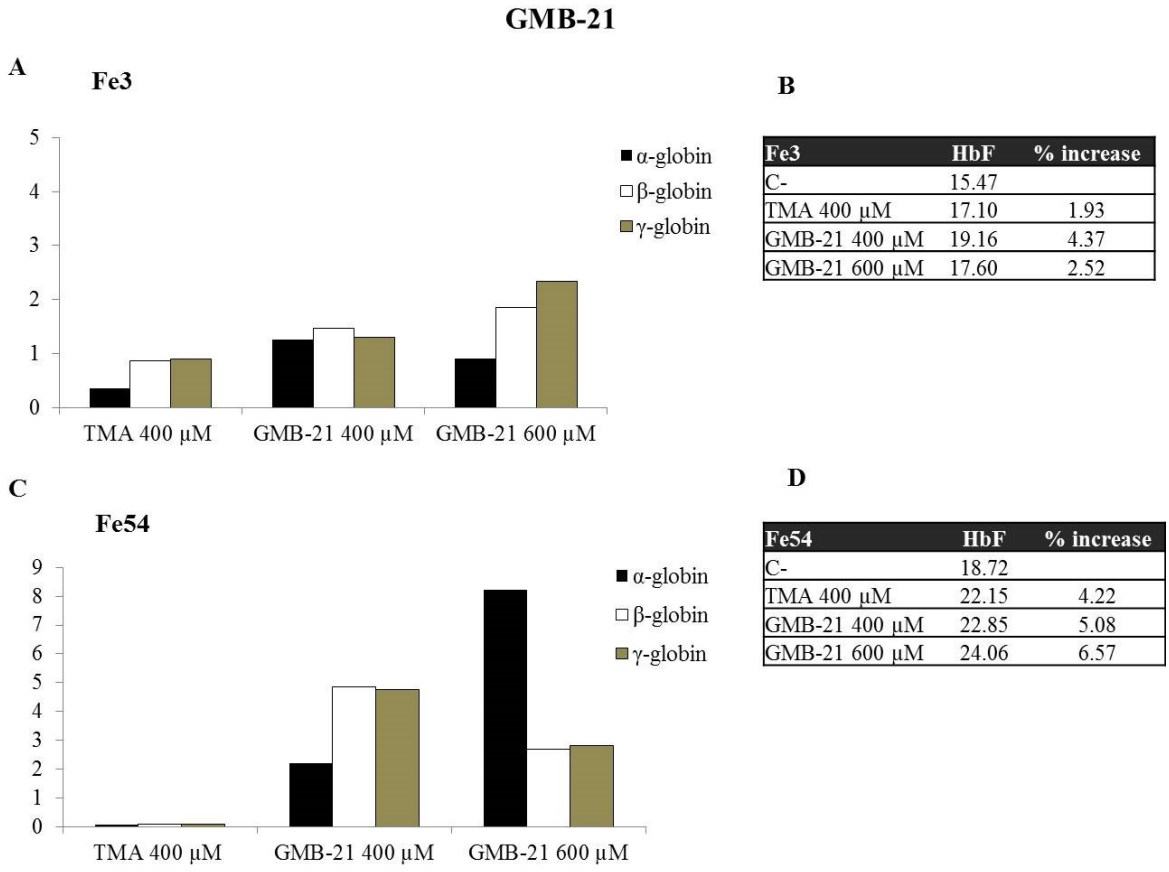
Fe3	HbF	% increase
C-	15.47	
TMA 400 µM	17.10	1.93
GMB-08 600 µM	18.87	4.02

Fe51	HbF	% increase
C-	12.14	
TMA 400 µM	10.36	-2.03
GMB-08 600 µM	17.95	6.61

Figure 42. Induction of globin genes expression (A, C) and HbF production (B, D) in GMB-08 treated cells. Treatments with GMB-08 600 µM produced a 3.5 fold increase of γ-globin mRNA in Fe51 (**C**). The percentage of increase of HbF was remarkable both in Fe3 (**B**) and Fe51 (**D**)

GMB-21

The analogue GMB-21 was tested in patients Fe3 ($\beta^0$39/β^+IVS I-110) and Fe54 ($\beta^0$39/β^+IVS I-110). The highest induction of γ-globin mRNA was achieved at 400 µM in Fe54 (4.76 fold), while a 2.34 fold and 2.80 fold induction were observed at 600 µM in Fe3 and Fe54 respectively. Fetal hemoglobin production was increased with GMB-21 at 400 µM and 600 µM, especially in Fe54 (Figure 43).



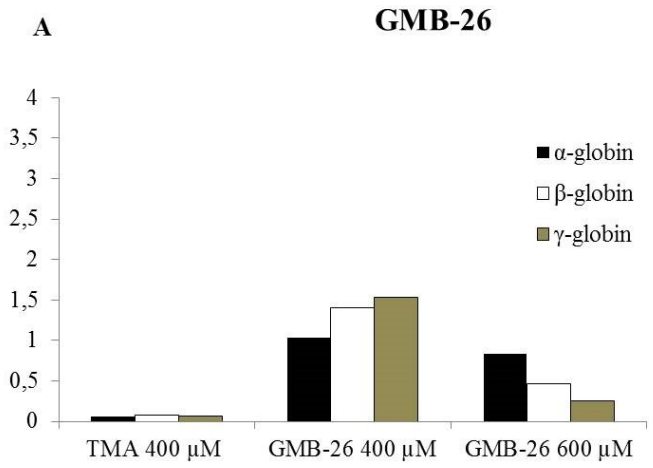
Fe3	HbF	% increase
C-	15.47	
TMA 400 µM	17.10	1.93
GMB-21 400 µM	19.16	4.37
GMB-21 600 µM	17.60	2.52

Fe54	HbF	% increase
C-	18.72	
TMA 400 µM	22.15	4.22
GMB-21 400 µM	22.85	5.08
GMB-21 600 µM	24.06	6.57

Figure 43. Increase of γ-globin gene expression (A, C) and HbF production (B, D) in GMB-21 treated cells. Treatments with GMB-21 400 µM produced the highest increase of γ-globin mRNA in Fe54 (**C**). A considerable increase of HbF was gained in Fe54 treated with GMB-21 600 µM.

GMB-26

The effects of GMB-26 on erythroid precursor cells was examined in Fe54 (Figure 44). The compound exhibited the most important induction of HbF than the other analogues; in fact the percentage of 8.39% increase was reaches with GMB-26 400 µM, despite the low level of induction of γ-globin transcripts.



B

Fe54	HbF	% increase
C-	18.72	
TMA 400 µM	22.15	4.22
GMB-26 400 µM	25.54	8.39
GMB-26 600 µM	19.09	0.46

Figure 44. Effects of GMB-26 on erythroid precursors cells. Treatments with GMB-26 400 µM produced the highest percentage increase of HbF (**B**), although no significant induction of γ-globin gene expression were detected.

3.4. DISCUSSION

Natural psoralen were investigated as potential novel fetal hemoglobin inducers, since it has been demonstrated that Angelicin is able to induce fetal hemoglobin production in human erythroid progenitors (Lampronti et al., 2003). In order to ameliorate biological activity and to reduce possible side effects (e.g. cytotoxic effects), other synthetic psoralen-like derivatives , such as TMA (4,6,4' –trimethylangelicin) have been evaluated. Recently, the research has been carried on testing 38 angelicin derivatives, called GMB, synthesized by Prof. Adriana Chilin (Department of Pharmaceutical and Pharmacological Sciences of Padua, Italy). The screening started from preliminary experiments on the human leukemic K562 cell line, in order to identify the molecules able to induce erythroid differentiation without undesirable cytotoxic effects. Four analogues (GMB-06, GMB-08, GMB-21, GMB-26) have been selected for further analysis on erythroid precursors cells from β-thalassemic patients, as they differentiate more than 20% of K562 cells. The selected compound displayed no antiproliferative effects on erythroid human progenitors. GMB-06 was active at all concentration tested, leading to an increase in γ-globin mRNA; HPLC analysis of cells lysates pointed out a maximum of 3.61% increase of HbF content in GMB-06 treated cells. After the treatment of erythroid precursors of Fe51 with GMB-08 600 µM, a considerable induction of γ-globin gene expression was correlated with a 6.61%

increase of fetal hemoglobin. GMB-21 exhibited dose depending induction of fetal hemoglobin production in Fe51. Finally, with GMB-26 the percentage of 8.39% increase was reaches in Fe54. In conclusion, the four angelicin analogues might be used for further investigation in a larger samples of β-thalassemic patients with different genotype, aimed at identifying novel therapeutic agents for a better management of β-thalassemia providing a personalized therapy.

Part II:

Delivery of PNA and therapeutic applications in erythroid cells

1. PEPTIDE NUCLEIC ACID (PNA)

1.1. INTRODUCTION

Peptide nucleic acid (PNA) is an organic polymer similar to DNA and RNA developed by Nielsen P. et al. in 1991 as a ligand for the recognition of specific sequences on double-stranded DNA, via Hoogsteen base pairing in the major groove (Nielsen et al., 1991). Unlike DNA or DNA analogs, PNAs do not contain any phosphate groups or (pentose) sugar moieties, since the deoxyribose-phosphate or ribose-phosphate backbone are replaced with repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The various purine and pyrimidine bases are linked to the backbone by methylene carbonyl bonds (Figure 45) (Egholm et al., 1993). The backbone of PNA is acyclic, achiral and neutral, it is chemically stable and resistant to hydrolytic (enzymatic) cleavage and thus not expected to be degraded inside a living cell. Because of their stability, peptide nucleic acids are ideal candidates as antisense or antigene therapeutic agents and are currently used as powerful tools in molecular biology and in diagnostics.

1.1.1. Structure and chemical properties of PNAs

As already mentioned, the molecule of PNA consists of a skeleton of repeating units of N-(2-aminoethyl)-glycine linked together by peptide bonds. Purine and pyrimidine bases are linked to this backbone by methylene-carbonyl bonds (Figure 45). The procedures for PNA synthesis are similar to those employed for peptide synthesis, using standard solid-phase manual or automated synthesis.

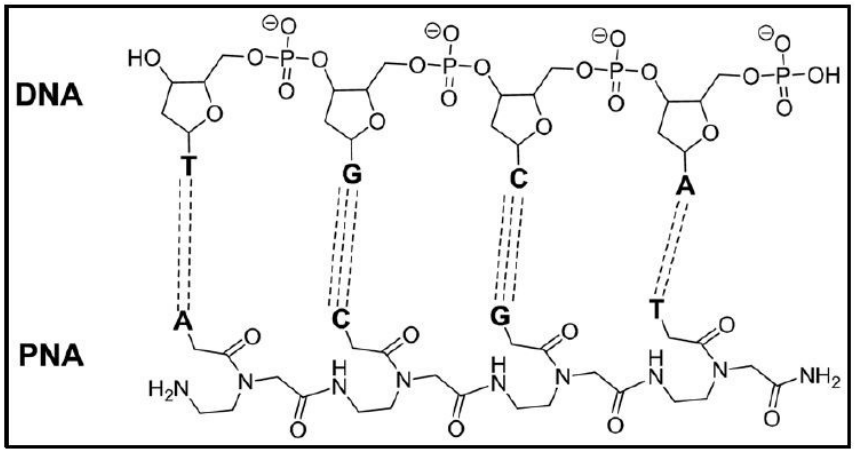


Figure 45. Chemical structure of DNA and PNA.

In comparison with normal DNA and RNA oligonucleotides, PNAs are very resistant to nuclease digestion since the nucleases cannot digest their altered backbones. Studies indicate that PNAs are stable inside cells for at least 48 h, while the half-life for most of the unmodified DNA and RNA oligonucleotides is approximately 15 min or shorter (McMahon et al., 2002). PNAs are also stable to protease digestion. PNAs are not recognized by polymerases and therefore cannot be directly used as primers or be copied.

PNAs can bind to complementary nucleic acids in both antiparallel and parallel orientation with high specificity and selectivity, forming Watson–Crick base pairs, even if the antiparallel orientation is strongly preferred. PNAs hybridize to complementary DNA and RNA sequences with high specificity and selectivity, leading to PNA–RNA and PNA–DNA hybrids that are more stable than the corresponding nucleic acid complexes (Egholm et al., 1993) This is a consequence of the neutral character of the PNA backbone that leads to the lack of charge repulsion between the PNA strand and the DNA strand. The stability of the PNA-DNA or PNA–RNA duplex has been evaluated by comparison of the thermal melting temperature (Tm), defined as the temperature at which 50% of the complexes have been dissociated. The thermal stability of a PNA–RNA duplex is even higher than that of a PNA–DNA complex (Jensen et al., 1997). PNA-DNA hybridization is significantly affected by base mismatches and PNA can maintain sequence discrimination up to the level of a single mismatch. A 15-mer PNA were used in order to test all possible single mismatch combinations in both PNA/DNA duplexes and corresponding DNA/DNA

duplexes. In the PNA/DNA duplexes, Tm was 15°C, whereas it was 11°C in the corresponding DNA/DNA duplexes (Nielsen et al., 1999).

PNA binding modes to either single-stranded DNA or RNA, or to double-stranded DNA have been analyzed by nuclear magnetic resonance and by X-ray crystallography (Rasmussen et al., 1997). A high cytosine content of the PNA appeared to be required in the classical triplex formation (Figure 46A). This PNA-DNA-DNA triplexes are much less stable than the corresponding triplex invasion complexes, but it were the original aim of the PNA design. The preferred strategy of homopyrimidine PNAs to target double stranded DNA is through triplex strand invasion. According to this binding mode, homopyrimidine PNAs with a minimum of 10-mers, as well as PNAs containing a high proportion of pyrimidine residues, bind to complementary DNA sequences to form highly stable (PNA)2–DNA triplex helices. In the resulting structure, called D-loops, one PNA strand hybridizes to DNA through standard Watson–Crick base pairing rules, while the other PNA strand binds to DNA through Hoogsteen hydrogen bonds (Figure 46B). Other binding modes for PNA have, however, been demonstrated: very purine rich PNAs form extremely stable PNA-DNA duplexes through standard duplex invasion (Figure 46C), while PNAs containing modified nucleobases form very stable double duplex invasion complexes (Figure 46D) (Pellestor et al., 2004; Nielsen et al., 1999).

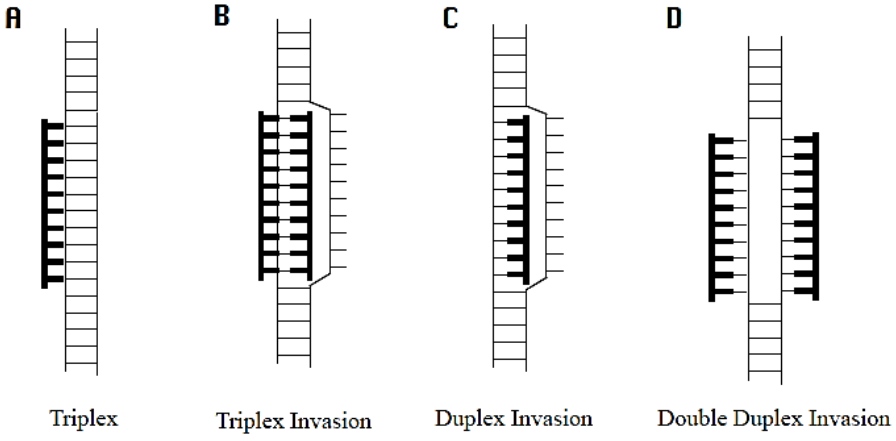


Figure 46. Schematic representation of PNA binding modes for targeting double stranded DNA. PNAs are depicted as thick structures. (**A**) triple helical structure (triplex); (**B**) triplex strand invasion, leading to the displacement of the second DNA strand into a 'D-loop'; (**C**) standard duplex invasion; (**D**) very stable double-duplex invasion.

1.1.2. Therapeutic application of PNAs

Thanks to their high stability and their resistance to nuclease and protease, PNAs appear to be valid alternative molecules to regulate gene expression both *in vivo* than *in vitro*. It has been also demonstrated that a double-stranded decoy molecules based on PNA–DNA chimeras was resistant to exonucleases (both 3′→5′ and 5′→3′ exonucleases), endo-nucleases and 5′-phosphatases (Borgatti et al., 2003). Further experiments highlighted that using liposomes as protectin agents, PNA-based molecules were more resistant than DNA-based decoys to exo- and endo-nucleases, serum and cellular extracts (Gambari et al., 2010). Other studies report that PNAs are very stable molecules in cell culture conditions and mostly in biological fluids (Demidov et al., 1994).

PNAs are able to target RNAs, through a steric hindrance of translation. For an efficient recognition, the localization of the PNA must coincide with the target RNA. Several studies report that PNA tends to accumulate in endocytic compartment and in nuclei (Folini et al., 2003; Bonham et al., 1995). Many successful antisense PNAs have been reported using cell-culture systems, including PNAs targeted against, the mdm2 oncogene (Shiraishi et al., 2004) and isoform-selective inhibition of caveolin-1 (Liu et al., 2004). In a mouse model of familial Amyotrophic lateral sclerosis (ALS), PNA-mediated inhibition of glutamate receptor mRNA and delayed disease onset was demonstrated after repeated intraperitoneal administration (Rembach et al., 2004). Moreover, antisense PNAs have been developed in order to inhibit mRNA splice site selection, for instance PNA oligomers targeted to a splice site that leads to a mature mRNA coding for a membrane-bound form of the interleukin-5 receptor (Karras et al., 2000). PNAs may also interfere with other cellular processes: PNAs against the RNA of telomerase are efficient inhibitors of this enzyme (Norton et al., 1996), PNA targeted to HIV RNA inhibits reverse transcription of this RNA (Koppelhus et al., 1997), and PNA targeted to certain sequences of ribosomal RNA are general inhibitors of translation and show bacteriostatic activity (Good et al., 1998). An interesting study by Wang et al. reported that a PNA introduced via electroporation was able to increase the transcription of γ-globin mRNA in human erythroleukemia cell line K562. This PNA targeted the -280 region of the gene, which is important for binding the transcription factor Oct-1, a repressors of γ-globin gene (Wang et al., 1999). Antigene peptide nucleic acid (PNA) has been described for selective inhibition of MYCN transcription in neuroblastoma cells, leading to cell growth inhibition (Tonelli et al., 2005). PNA–DNA chimeras have been reported as reagents of great interest in gene

therapy, as they are able to stably interact with TFs inhibiting TF-dependent effects (Borgatti et al., 2004). More recently, PNAs have been developed in order to target miRNAs and modify their biological metabolism within the cells. An antimiR-210 PNA reduced the level of miRNA-210 in K562 cells treated with Mithramycin, resulting in a decrease in γ-globin mRNA and lower expression of differentiated functions (Fabbri et al., 2011).

The high-affinity binding of PNA oligomers has led to the development of other applications of PNA, for istance as a diagnostic probe for detecting genetic mutations, as a tool in molecular biology to enhance PCR amplification and as a probe for nucleic acid biosensor (Ray et al., 2000).

1.1.3. Modified PNAs to improve cellular uptake

The poor cellular uptake of PNAs has been a major problem of practical applications of these oligomers. In order to use standard oligonucleotide transfectants as Lipofectamine, PNA, which has a neutral backbone, needs to be hybridized to complementary oligodeoxynucleotide (ODN) that aids the electrostatic complexation with the positively charged lipids (Braasch et al., 2002). The most popular approach to enhance cellular delivery has been conjugation of PNA with cell penetrating peptides that deliver the conjugate through the endocytosis pathway (Hassane et al., 2010). PNA monomers, having the backbone functionalized with amino acid side chains, have been largely explored. PNA with short oligolysine enabled efficient delivery in various cell lines and different studies demonstrated that PNA conjugated at the carboxyl terminus with octaarginine was efficiently taken up in human leukemic K562 cells and human gliomas cell lines U251, U373 and T98g (Fabbri et al., 2011; Brognara et al., 2014). Negatively charged moieties such as sulfate and phosphonate installed on the PNA backbone allowed delivery of PNA by standard cationic liposomes (Shiraishi et al., 2008). Nevertheless, optimization of cellular delivery of PNA is still a vigorous and important area of research. For this reason, Prof. Alessandra Romanelli (Department of Pharmacy, University of Naples) developed a liposomes formulation loaded with PNA, that we have tested in our research group.

2. LIPOSOME-MEDIATED DELIVERY OF PNA IN ERYTHROID CELLS

Liposomes have been successfully applied for the in vivo delivery of drugs and contrast agents (Riche et al., 2004; Lao et al., 2013); several liposomal formulations have been approved by FDA and are employed in clinic for drug delivery in the cure of various diseases ranging from cancer (Doxil®), to fungal infections (Ambisome®) to age related macular degeneration (Visudyne®) (Zhang et al., 2013). The composition of the liposome, which determines its structural properties (size, external charge, polydispersity index, transition temperature, drug loading and release), its stability in the biological fluids and also the ability to circulate for long times, is chosen based on the molecule that has to be encapsulated. In order to find out a novel delivery system of PNA, we have investigated the encapsulation in a liposomal composition, based on the zwitterionic egg PC, cholesterol and DSPE-PEG2000. The PNA sequences were designed to be complementary to miR-210 a noncoding RNA, associated with erythroid differentiation of leukemic K562 cells. We have verified the uptake of the liposomes in K562 and the ability of the formulation to down-regulate the target miR-210.

2.1. MATERIAL AND METHODS

2.1.1. Culture of human K562 erythroleukemia cells

K562 cells were cultured in humidified atmosphere of 5% CO_2, in Roswell Park Memorial Institute RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 50 units/ml penicillin (Lonza, Verviers, Belgium) and 50 μg/ml streptomycin (Lonza, Verviers, Belgium), as previously describe on page 31.

Cells were counted with the BECKMAN COULTER® Z2 (Beckman, Pasadena, California, USA), in the proper range (8-20 μm for K562 cells).

2.1.2. PNA uptake by K562 cells

To determine the PNA uptake, flow cytometry analyses (BD FACScan) were performed. In order to observe uptake kinetics, K562 cells were seeded at initial concentrations of 3 x 10^5 per well in 24-well plates for 4, 24 and 48 hours and incubated with PNA-a210, PNA-a210-R8 or LIPO-PNA-a210 at final concentrations of 2μM and 4μM. After the treatment, cells were centrifuged at 1800 rpm for 5 minutes, washed in PBS (Lonza BioWhittaker, Basel, Switzerland) with 1% FBS and suspended in 200μl PBS with 1% FBS. For each sample, 30000 events were analysed and fluorescence emission at 515-545 nm (FL1) was detected, using BD Cell Quest Pro. The intracellular distribution of FLUO-LIPO-PNA-a210 was studied at first by microscopy analysis of living cells using the BioStation instrument, a compact cell incubation and monitoring system (BioStation IM, Nikon Instruments Europe B.V., Italy), previously demonstrated to be a suitable system to detect intracellular distribution of fluorescein-labelled PNAs (Brognara et al., 2014). Then, the cells were fixed, counterstained with 4α,6-diamidino-2- phenylindole (DAPI) for nuclear labelling and further analysed. Live images of cells treated with FLUO-PNA-a210 and Rho-LIPOFLUO-PNA-a210 were also obtained using a Nikon Swept Field Confocal equipped with CFI Plan Apo VC60XH objective (numerical aperture, 1.4) (Nikon Instruments, Melville, NY, USA) and an Andor DU885 electron multiplying charge- coupled device (EM-CCD) camera (Andor Technology Ltd, Belfast, Northern Ireland). Acquired images were then analyzed by using open source software Fiji (Franceschini et al., 2015; Venco et al., 2015).

2.1.3. Transfection protocols for standard RNA antagomiR

The transfection of the anti-microRNAs was carried out following the protocol reported by Ambion (Applied Biosystems, Foster City, CA, USA) and described elsewhere (Bianchi et al., 2015). Treatments with increasing concentrations of antagomiR-210 (AM-105116 Applied Biosystems, Foster City, CA, USA) were performed using the siPort NeoFX transfection reagent.

2.1.4. Quantitative analyses of miRNAs

For miRNA quantification RNA was isolated from K562 cells treated or not with the PNAs and reverse transcriptase (RT) reactions were performed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City,CA, USA). Real-time PCR was performed using the specific human TaqMan Micro RNA Assay Kits from Applied Biosystems for hsa-miR-210 and hsa-miR-221. 500 ng per sample were used for the assays and all RT reactions, including no-template controls and RT-minus controls, and the analysis were performed in duplicate using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Relative expression was calculated using the comparative cycle threshold method and as reference U6 snRNA (TM:001973) were used to normalize all RNA samples, since it remains constant in the assayed samples by miRprofiling and quantitative RT-PCR analysis, as previously reported (Brognara et al., 2012).

2.1.5. Statistical analysis

All the data were normally distributed and presented as mean ± S.D. Statistical differences between groups were compared using one-way ANOVA (ANalyses Of VAriance between groups) software. Statistical differences were considered significant when $p< 0.05$ (*), highly significant when $p< 0.01$ (**).

2.1.6. Liposome preparation and structural characterization

The procedure was carried out in the laboratory of Prof. Romanelli as described in our work (Avitabile et al., 2015). After their preparation, PNA-antimiR210 and FLUO-PNA-antimiR210 derivatives were encapsulated in mixed egg PC/Chol/DSPE-PEG2000 liposomes at 47/47/6 molar ratio. PNAs molecules were loaded exploring three different methods: thin film method/sonication (a), freeze/thawing (b) and SNALPs (c). Empty liposomes were also prepared according to these procedures. Moreover, any substantial difference has been revealed in the encapsulation efficiency achieved with the three procedures The sequences of the PNAs used in this study are reported in Table 14.

Liposomes labelled with rhodamine encapsulating the FLUO-PNA-antimiR210 (Rho-LIPO-FLUOPNA-a210, were also prepared and employed to detect the cellular uptake of PNA-a210 in K562 cells by confocal microscopy. The freeze/thawing method was found to yield lower polydispersity indexes (PDI) of the liposomes, as compared to the other methods, and was therefore employed to encapsulate PNA oligomers for in vitro studies. A further advantage of this method is that it permits to avoid the presence of traces of ethanol required during the SNALPs preparation, which are hardly removed and may be toxic to cells. In Figure 47 is depicted a schematic representation of the freeze/thawing encapsulation method.

Table 14. Sequences of the PNAs employed in the study.

PNA	SEQUENCE
PNA-a210	H-CCGCTGTCACACGCACAG-NH_2
PNA-a210 scramble	H-AGCGACGCGATCCTCACC-NH_2
PNA-a210-R8	H-CCGCTGTCACACGCACAG-$(R)_8$-NH_2
FLUO-PNA-a210	FLUO-Ahx-CCGCTGTCACACGCACAG-NH_2
FLUO-PNA-a210-R8	FLUO-Ahx-CCGCTGTCACACGCACAG-$(R)_8$-NH_2

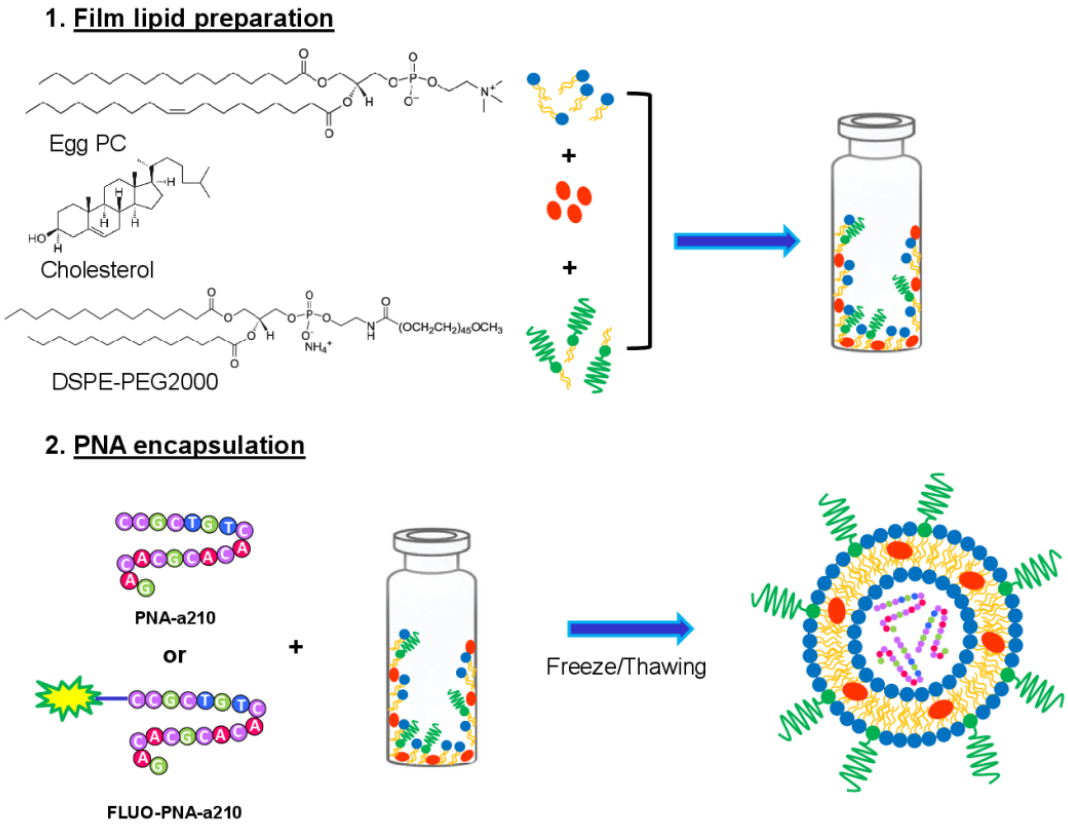


Figure 47. Schematic representation of the freeze/thawing encapsulation method. In the first step the mixture of lipids was dried under vacuum to form a lipid film, then the PNA solution is added to the vial and liposomes encapsulating PNA are obtained (From Avitabile et al., *Bioconjug Chem* 2015).

2.2. RESULTS

2.2.1. Uptake of PNAs by K562 cells

Uptake studies were carried out employing K562 cells as experimental model system, using the following fluorescently labelled molecules: FLUO-PNA-a210, FLUO-PNA-a210-R8 (a PNA targeting miR-210 and conjugated to a polyarginine R8 sequence) and the LIPO-FLUO-PNA-a210 formulation. Figure 48 shows a first set of experiments in which K562 cells were cultured for 4 hours in the presence of 2μM and 4 μM FLUO-PNA-a210, FLUO-PNA-a210-R8 and LIPO-FLUO-PNA-a210 and were analyzed by FACS.

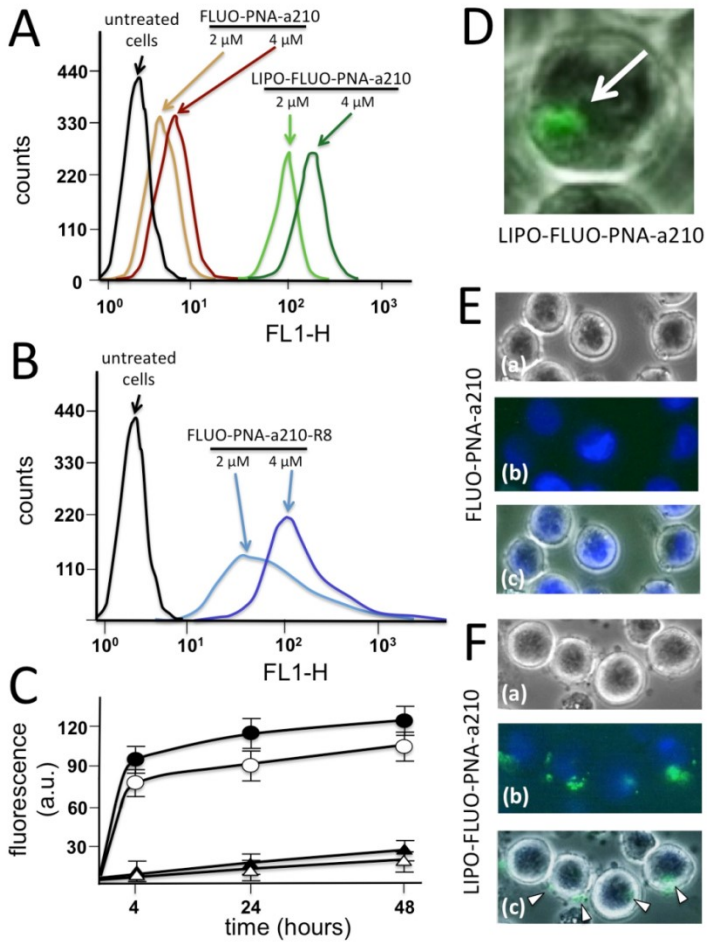


Figure 48. Uptake of PNAs. **A**,**B**. Representative FACS analysis of K562 cells cultured for 4 hours in the presence of the indicated concentrations of FLUO-PNA-a210 (**A**), LIPO-FLUO-PNA-a210 (**A**) and FLUO-PNA-a210-R8 (**B**). **C**. Kinetics of uptake of 2 μM FLUO-PNA-a210 (open triangles), 4 μM FLUO-PNA-a210 (black triangles), 2 μM LIPO-FLUO-PNA-a210 (open circles), and 4 μM LIPO-FLUO-PNA-a210 (black circles). **D**. Representative microscopic analysis showing intracellular localization of LIPO-FLUO-PNA-a210 (arrowed) in K562 cells. **E**,**F**. Microscopic analysis of: a) K562 cells, phasecontrast analysis; b) K562 cells stained with DAPI and treated for 24 hours with 1 μM FLUO-PNAa210(E) and LIPO-FLUO-PNA-a210 (F), (c) merge. (From Avitabile et al., *Bioconjug Chem* 2015).

As expected, low uptake levels were found when FLUO-PNA-a210 was employed. This is perfectly in agreement with results previously published by our research group demonstrating that the PNA-a210 is internalized by target cells only if it is linked to a octarginine R8 peptide (Fabbri et al., 2011; Brognara et al., 2014). The summary of the time course of the uptake is shown in Figure 48, which indicates that uptake of LIPO-PNA-a210 is fast and a plateau level is reached between 4 and 24 hours culture period. No appreciable uptake of FLUO-PNA-a210 by K562 cells was detectable even after 48 hours of culture. The representative microscopic analyses shown in Figure 48 (panels D-F)

demonstrate that no evidence of intracellular uptake of FLUO-PNA-a210 is detectable. By contrast, liposomes carrying the FLUO-PNA-a210 bind to the cellular membrane and, when internalized, are located mostly in the intracellular matrix (Figure 48, D and F). When staining of nuclei is performed (Figure 48 F, b), we can conclude that no liposome-mediated delivery of FLUO-PNA-a210 is detectable at nuclear level, and the localization is mostly cytoplasmic. Furthermore, to conclusively demonstrate intracellular localization of LIPO-PNA-a210, confocal microscopic analysis was performed on living cells (Figure 49). Figure 49A shows that, in agreement with the FACS data shown on Figure 48A and the microscopic analysis shown in Figure 48E, no uptake of FLUO-PNA-a210 is detectable, since almost all of the fluorescence was found outside the cells. On the contrary, when rhodamine labelled liposomes carrying FLUO-PNA-a210 (Rho-LIPO-FLUO-PNA) were employed, most of the fluorescence was found within the cells, formally demonstrating liposome-mediated uptake of the PNA-a210 (Figure 49B). In any case, these results were considered a promising background to investigate possible effects on miR-210 in LIPO-FLUO-PNA-a210 treated cells. Taken together, the data shown in Figure 48 and Figure 49 strongly suggest that the liposomes facilitate cellular binding and uptake of FLUO-PNA-a210 molecules.

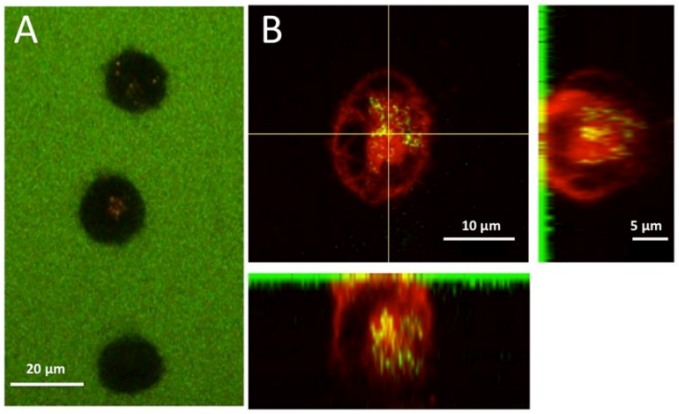


Figure 49. **Uptake of PNA: confocal microscopy analyses. A**. Representative confocal microscopic analysis of K562 cells cultured in the presence of FLUO-PNA-a210 (24 hours, 1 µM). **B**. Representative live images of K562 cells after 24h incubation with 1 µM Rho-LIPO-FLUO-PNAa210. Fluorescence profile was run across the x-y axes of the cell body and orthogonal view of merged image was obtained with confocal Z-stack (From Avitabile et al., *Bioconjug Chem* 2015).

2.2.2. Effects of the liposome-delivered PNA-a210 on the growth of K562 cells

We had determined at first the effects of the different treatments (48 hours incubation) on cell growth (Figure 50). K562 cells were treated with different concentration of LIPO, LIPO-PNA-a210, LIPO-PNA-a210-scr, PNA-a210, PNA-a210-R8 and antagomiR-210. Interestingly, the LIPO and the LIPO-PNA-a210 preparations caused only minor effects on the rate of cell growth, demonstrating lack of cytotoxic activity.

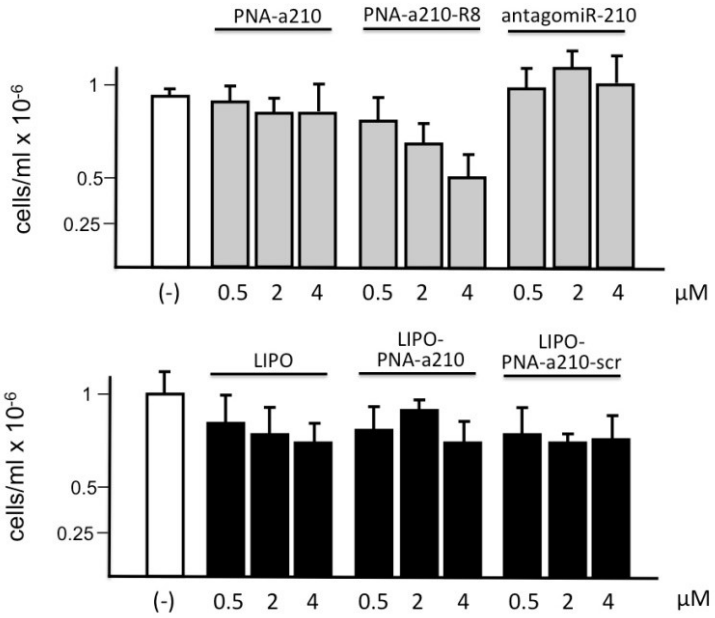


Figure 50. Effects of the different treatments on cell growth. K562 cells were treated for 4 days with scalar doses of PNA-a210, PNA-a210-R8, antagomiR-210 (upper panel), LIPO, LIPO-PNA-a210 and LIPO-PNA-a210-scr (lower panel). (From Avitabile et al., Supplemetary Material *Bioconjug Chem* 2015).

2.2.3. Effects of LIPO-PNA-a210 on miR-210 in K562 cells.

In order to determine whether the exposure to LIPO-PNA-a210 leads to alteration of miR-210 in K562 cells, RNA isolated from treated cells was used for RT-qPCR reactions amplifying miR-210. When K562 cells were cultured in the presence of LIPO-

PNA-a210 a very reproducible effect was obtained, as shown in Figure 51. The results demonstrate that the miR-210 specific hybridization signal was reduced when RNA was isolated from K562 cells cultured for 48 h in the presence of LIPO-PNA-a210 (Figure 51A). On the contrary, the PNA-a210, lacking the R8 peptide, was not effective, in agreement with elsewhere published results (Fabbri et al., 2011). Notably, no effects were found using the liposomes carrying a scrambled PNA (LIPO-PNA-a210-scr) (Figure 51A). As positive controls we employed the elsewhere validated PNA-a210-R8 and a commercially-available antagomiR-210 (Fabbri et al., 2011; Bianchi et al., 2015). The results obtained are shown in Figure 51B, and they support the concept that the LIPO-PNA-a210 formulation approaches the activity of these already validated anti-miR-210 reagents.

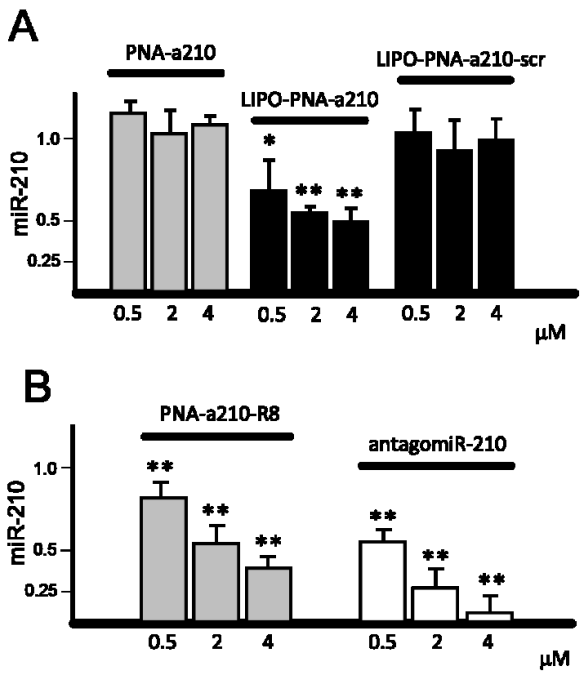


Figure 51. Real time detection of miR-210. K562 cells were treated for 48 hours with PNA-a210 (**A**), LIPO-PNA-a210 (**A**), LIPO-PNA-a210-scr (**A**), PNA-a210-R8 (**B**) and antagomiR-210 reference control (**B**), used at the indicated concentrations. RNA was extracted and RT-qPCR identifying miR-210 was performed and compared to control untreated K562 cells. Results represent the average ± S.D. of three independent experiments (*, $p<0.05$, significant; **, $p<0.01$, highly significant) (From Avitabile et al., *Bioconjug Chem* 2015)

2.2.4. Specificity of the effects of LIPO-PNA-a210

As far as specificity, we verified this issue by analyzing the levels of an additional microRNA, miR-221, in K562 cells treated with LIPO-PNA- a210. As control a PNA-a221 and a PNA-a221-R8 were also employed, demonstrating as reported elsewhere (Brognara et al., 2014), an inhibitory activity of PNA-a221-R8, but not of PNA-a221 on PCR-mediated generation of miR-221 signals. The data conclusively demonstrated that the effects of LIPO-PNA-a210 were selective, as no major effects were obtained in treated cells on miR-221 (Figure 52).

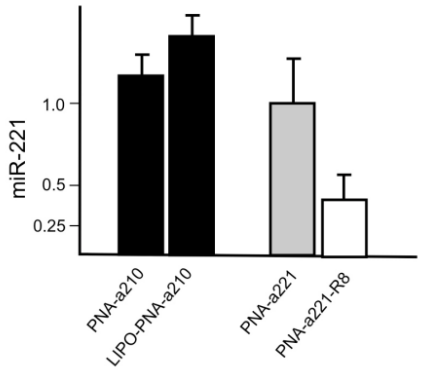


Figure 52. Real time detection of miR-221. K562 cells were treated for 48 hours with 2 μM of PNA-a210 and LIPO-PNA-a210 (black bars), PNA-a221(grey bar) and PNA-a221-R8 (white bar). RNA was extracted and RT-qPCR identifying miR221 performed and compared to control untreated K562 cells. The sequences of PNA-a221 and PNA-a221-R8, and conditions for miR-221 RT-qPCR conditions have been reported elsewhere (Brognara et al., 2014) (From Avitabile et al., Supplemetary Material, *Bioconjug Chem,* 2015)

2.3. DISCUSSION

Peptide nucleic acids (PNAs) have been largely used for their remarkable hybridization properties and their various applications. In fact, PNAs have been proposed as antigene (Tonelli et al., 2005) and antisense molecules (Shiraishi et al., 2004), as decoy molecules targeting transcription factor (Borgatti et al., 2004) as efficient tools in molecular biology and diagnostic and more recently in miRNA therapeutics (Fabbri et al., 2011). Efficient intracellular delivery is essential for high activity of peptide nucleic acid

and several strategies have been developed in order to enhance their cellular uptake. We have tested the first example of a liposomal PNA formulation, in which a PNA oligomer is efficiently loaded in a pegylated liposome composition based on the zwitterionic PC phospholipid, cholesterol and DSPE-PEG2000. The PNA sequences were designed to be complementary to miR-210. The liposomal formulation LIPO-PNA-a210 were compared to the elsewhere validated PNA-a210-R8. FACS analysis revealed a fast uptake of the liposomes carrying the PNA-a210 in K562 cell lines and the nuclei staining highlighted a mostly cytoplasmic localization of PNA. The confocal microscopic analysis confirmed the intracellular localization of LIPO-PNA-a210, while the PNA-a210 were detected only outside the cells. The liposomal formulation caused only minor effects on the rate of cell growth, while PNA-a210-R8 exhibited a cytotoxic activity at the highest concentration tested. The level of expression of miRNA-210 was reduced after the treatment with LIPO-PNA-a210 for 48h, as demonstrated by RT-qPCR analysis. Moreover, it has been demonstrated that the liposome-delivered PNA-a210 maintained its specificity on miR-210 downregulation, since the expression of the unrelated miR-221 were not affected by LIPO-PNA-a210. Our results indicates that, using liposome-delivered PNA a low concentration of the peptide is needed to trigger a biological effect. Therefore, further experiments are needed to compare liposome-delivered PNAs with R8-delivered PNAs and commercially available antagomiRs, including the use of different cell lines, the design of anti-miR molecules with different length, PNA sequences targeting different miRNAs, extensive studies on miRNA-regulated mRNAs.

In any case, the demonstration that PNA sequences can be delivered by liposomes open a novel strategy for selective targeting, since liposomes can be functionalized with antibodies (Allen et al., 1995), peptides (Ducat et al., 2010), aptamers (Song et al, 2015).

3. PNA TARGETING β-GLOBIN mRNA IN MURINE ERYTHROLEUKEMIA CELLS

Peptide nucleic acids have not yet been employed to inhibit the expression of globin genes in erythroid cells. Thus, we have verified whether PNAs targeting globin mRNAs can be used to modulate globin gene expression and to reduce the level of a given type of globin. A PNA targeting murine adult β-globin mRNAs and another recognizing the human γ-globin mRNAs were produced. Murine Erythroleukemia (MEL) cells were chosen as experimental model system since they express, after treatment with different inducers, almost adult hemoglobin. Hence, MEL cells are a reliable cellular "biosensor" for alteration of β-globin gene expression using the simple benzidine-test.

3.1. MATERIAL AND METHODS

3.1.1. Synthesis of PNAs

The peptide nucleic acids (PNAs) used in our experiments were synthesized by Prof. Roberto Corradini and his collaborators (University of Parma, Dept. of Organic and Industrial Chemistry) using standard automated Fmoc-based chemistry with HBTU/DIPEA coupling on a ChemMatrix resin loaded with Fmoc-Gly-OH as first monomer (loading 0.2 mmol/g, 5 μmol scale), on a Syro II peptide synthesizer, using commercially available monomers (Link Technologies, Bellshill, UK). As elsewhere demonstrated, in order to improve the cellular uptake, the PNAs were linked to a polyarginine tail (R8) (Brognara et al., 2014) and Fmoc-Arg (Pbf)-OH (Sigma-Aldrich, St. Louis, MO, USA) was used for octaarginine synthesis (Montagner et al 2014). The sequences of the PNAs employed in this study are reported in Table 15.

Table 15. Sequences of the PNAs employed in the study.

	Sequence
Anti-M-βglob-PNA	H-R_8-GAG GCA GAG GAT AGG TCT-Gly-NH_2
Anti-H-γglob-PNA	H-R_8-TCG CAA AGC TGT CAA-Gly-NH_2

3.1.2. Culture of murine erythroleukemia (MEL) cells and human K562 cells

MEL cells were cultured in humidified atmosphere of 5% CO_2, in Roswell Park Memorial Institute RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 50 units/ml penicillin (Lonza, Verviers, Belgium) and 50 μg/ml streptomycin (Lonza, Verviers, Belgium) (Rutherford et al., 1979). Erythroid differentiation and the high production of hemoglobins were induced by treatment with dimethylsulfoxide (DMSO, Sigma-Aldrich) and hexamethylene bisacetamide (HMBA, Sigma-Aldrich) (Gambari et al., 1978; Gambari et al., 1979; Rutherford et al., 1979). Stock solutions of HMBA were stored at -20˚C in the dark and diluted immediately before use. Treatment of the MEL cells with HMBA and DMSO was carried out by adding the appropriate drug concentrations at the beginning of the cultures (30,000 cells/ml were seeded). The K562-D5 cell line was employed as it produces, in addition to hemoglobin (Hb) Gower1 and hemoglobin (Hb) Portland, HbF and HbA (Salvatori et al., 2009).

3.1.3. Effects of Anti-M-βglob-PNA on cell growth and differentiation of MEL cells

In order to determine the effects of the treatments on the proliferation of the MEL cells, cell growth was monitored by determining the cell number/ml using the BECKMAN COULTER® Z2 (Beckman, Pasadena, California, USA) in the proper range (10-15 μm for MEL cells). Erythroid differentiated MEL cells containing hemoglobin were detected by specific reaction with a benzidine/hydrogen peroxide solution (0.2% benzidine in 5mol/l glacial acetic acid, 10% H_2O_2).

3.1.4. RNA isolation

The total cellular RNA was isolated by TRIZOL® Reagent (Sigma-Aldrich, St.Louis, Missouri, USA), as well described in Chapter “Materials and Methods” of Part 1 of this PhD Thesis (page 33) quantified by measuring the absorbance at 260 nm (A260) in a spectrophotometer, and quality controlled by electrophoresis on 1% agarose gel.

3.1.5. Reverse transcription reaction- Random Hexamer

Reverse transcription of 500 ng of total RNA was performed using with the TaqMan® Reverse Transcription Reagents PCR kit (Applied Biosystems), as well described in Chapter "Materials and Methods" of Part 1 of this PhD Thesis, page 34.

3.1.6. Real Time quantitative-PCR analysis

Quantitative PCR assays were carried out using gene-specific double-quenched probes containing a 5'-FAM fluorophore, a 3'-IBFQ quencher and an internal ZEN quencher. The nucleotide sequences used for the RT-qPCR analysis of mouse globin mRNAs are reported in Table 16.

Table 16. **Sequences of primers and probes used in quantitative PCR reactions.**

MURINE primer/probe	SEQUENCE
α-globin forward	5'-CTG ACC TCC AAG TAC CGT TAA G-3'
α-globin reverse	5'-GCT TCT TCC TAC TCA GGC TTT AT-3'
α-globin probe	5'-/56-FAM/TCT CTC CCT/ZEN/TGC ACC TGT ACC TCT/3IABkFQ/-3'
β-globin forward	5'-GGA AAG GTG AAC TCC GAT GAA-3'
β-globin reverse	5'-TGA TAG CAG AGG CAG AGG ATA G-3'
β-globin probe	5'-/56-FAM/CCT TGG ACC/ZEN/CAG CGG TAC TTT GAT/3IABkFQ/-3'

The primers and probes used to assay mouse globins were purchased from Integrated DNA Technologies (IDT; Coralville, IA, USA). The relative expression was calculated using the comparative cycle threshold method and the endogenous control mouse gene, GAPDH, was used as a reference gene (PrimeTime Mm.PT.39a.1, IDT).

3.1.7. High performance liquid chromatography (HPLC) analysis

The cells were harvested, washed once with PBS and the pellets were lysed in lysis buffer (sodium dodecyl sulphate 0.01%). After spinning for 1 min in a microcentrifuge, the supernatant was collected and stored at 4˚C. Hemoglobins in the lysates were separated by cation-exchange HPLC (Pharmacia LKB Gradient Pump 2249, VWM 2141), using a Synchropak CM300 (250x4.6 mm) column (Eichrom Technologies, Inc., Darien, IL, USA) and BisTris (30 mM) buffer.

3.1.8. Bioinformatics analysis

The secondary structure of the mouse β^{major}- and β^{minor}-globin mRNA sequences, 5' untranslated region (UTR), coding sequence (CDS) and 3'UTR, was predicted using the program available online TBI ViennaRNA Web Services (http://rna.tbi.univie.ac.at). The mouse β-globin reference sequences analyzed were obtained from the NCBI website.

3.1.9. Statistical analysis

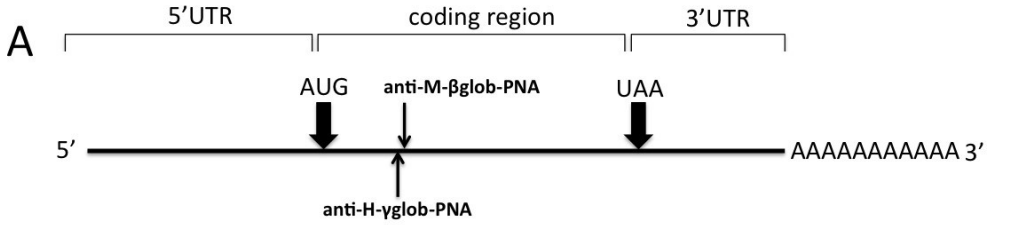
The results are expressed as the means ± standard error of the mean (SEM). Comparisons between groups were made using a paired Student's t-test and one-way analysis of variance (ANOVA). Statistical significance was defined with $p<0.01$.

3.2. RESULTS

3.2.1. Design of PNAs

The anti-M-βglob-PNA designed were able to hybridize to a region of both mouse β^{major}- and β^{minor}-globin mRNAs exhibiting similar predicted secondary structures as depicted in Figure 53B and C. This feature allows us to study the effects of the PNAs simply analyzing the proportion of erythroid differentiated MEL cells. These cells, upon the induction of erythroid differentiation with HMBA or DMSO produce almost

exclusively Hb^{major} ($\alpha_2\beta_2^{major}$) and Hb^{minor} ($\alpha_2\beta_2^{minor}$). Moreover, these PNAs display 4 to 8 mismatches with human β-globin (8 mismatches), γ-globin (3 mismatches) and ε-globin (4 mismatches) (Figure 53A, bottom panel). This allows us to verify possible non-specific inhibitory effects when used on K562 cell clones subjected to erythroid differentiation which produce, upon treatment with Mithramycin (MTH), high levels of Hb Gower 1 ($\zeta_2\varepsilon_2$), Hb Portland ($\zeta_2\gamma_2$), HbF ($\alpha_2\gamma_2$) and HbA ($\alpha_2\beta_2$) (Salvatori et al., 2009). Conversely, as control PNA molecules we used anti-H-γglob-PNA recognizing the evolutionarily homologue human γ-globin mRNAs and displaying 3 mismatches with the murine β^{major} and β^{minor} mRNAs (Figure 53A).



PNA	Sequence	Complementarity with potential globin mRNA targets				
		Mouse β^{major}	Mouse β^{minor}	Human ε	Human γ	Human β
anti-M-βglob-PNA	H-R$_8$-GAG GCA GAG GAT AGG TCT-Gly-NH$_2$	18/18	18/18	14/18	15/18	10/18
anti-H-γglob-PNA	H-R$_8$-TCG CAA AGC TGT CAA -Gly-NH$_2$	12/15	12/15	14/15	15/15	10/15

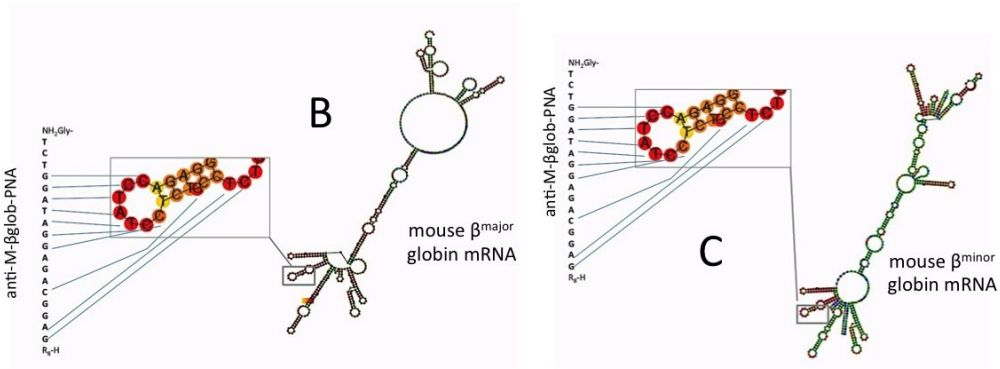


Figure 53. **(A) Location of the binding sites (upper panel) and sequences of the PNAs employed in our study (lower panel). (B and C) Possible interactions between anti-M-βglob-PNA and mouse (B) β^{major} and (C) β^{minor} globin mRNAs.** Predicted secondary structure of the 5'UTR, CDS and 3'UTR region of mouse β^{major}- and β^{minor}-globin mRNAs were based on the NCBI website references sequences, NM_001278161.1 and NM_016956.3, respectively and obtained using the TBI ViennaRNA Web Services (http://rna.tbi.univie.ac.at/). Magnification of the central portion of the CDS site of the globins points out the possible interaction between the β-globin CDS target strands and the anti-M-βglob-PNA region (From Montagner et al., *Int J Mol Med,* 2015).

3.2.2. Effects of the anti-M-βglob-PNA on the growth of MEL cells

The kinetics of differentiation and cell growth obtained when the MEL cells are treated with 2% DMSO or 2.5 mM HMBA are shown in Figure 54. The high level of induction (>80% after 3 or 4 days in all the experiments performed) confirms that this cellular system is excellent to determine inhibitors of the expression of adult β-globin genes, since, unlike other erythroid cellular model systems (such as human K562 cells), these cells mostly produce the adult-type Hb^{major} and Hb^{minor} hemoglobins (Rutherford et al., 1979). As shown in Figure 54C, the addition of anti-M-βglob-PNA did not alter the proliferation rate of these cells, formally demonstrating no cytotoxic effects of this PNA on the MEL cells, either in the absence of differentiation inducers (data not shown) or in the presence of DMSO or HMBA.

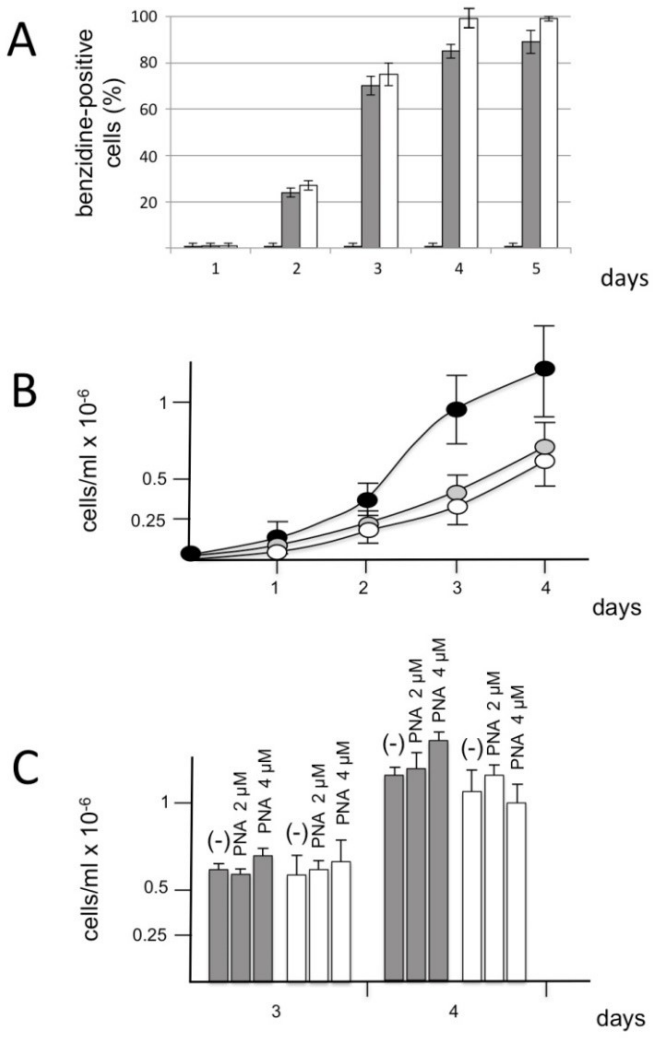


Figure 54. **Kinetics of differentiation (A) and cell growth (B,C) of treated MEL cells.** (**A**) Level of erythroid differentiation obtained when murine erythroleukemia (MEL) cells are treated with 2% dimethylsulfoxide (DMSO) (grey boxes), or with 2.5 mM hexamethylene bisacetamide (HMBA) (white boxes). Untreated cells, black boxes. (**B**) Proliferation of MEL cells cultured without inducers (black symbols), or in the presence of 2% DMSO (grey symbols), or 2.5 mM HMBA (white symbols). (**C**) Effects of 2 and 4 µM of anti-M-βglob-PNA on the proliferation of MEL cells treated with 2% DMSO (grey boxes), or with 2.5 mM HMBA (white boxes) (From Montagner et al., *Int J Mol Med*, 2015).

3.2.3. Effects of anti-M-βglob-PNA inhibits on the erythroid differentiation of MEL cells induced by DMSO and HMBA

The effects of anti-M-βglob-PNA on the erythroid differentiation of MEL cells were first analyzed by benzidine staining of the treated cells. The results confirmed a decrease in the proportion of benzidine-positive (hemoglobin-containing) PNA-treated cells. Figure 55 clearly illustrates that the MEL cells treated with DMSO (Figure 55G and H) and HMBA (Figure 55C and D) do not efficiently differentiate in the presence of anti-M-βglob-PNA.

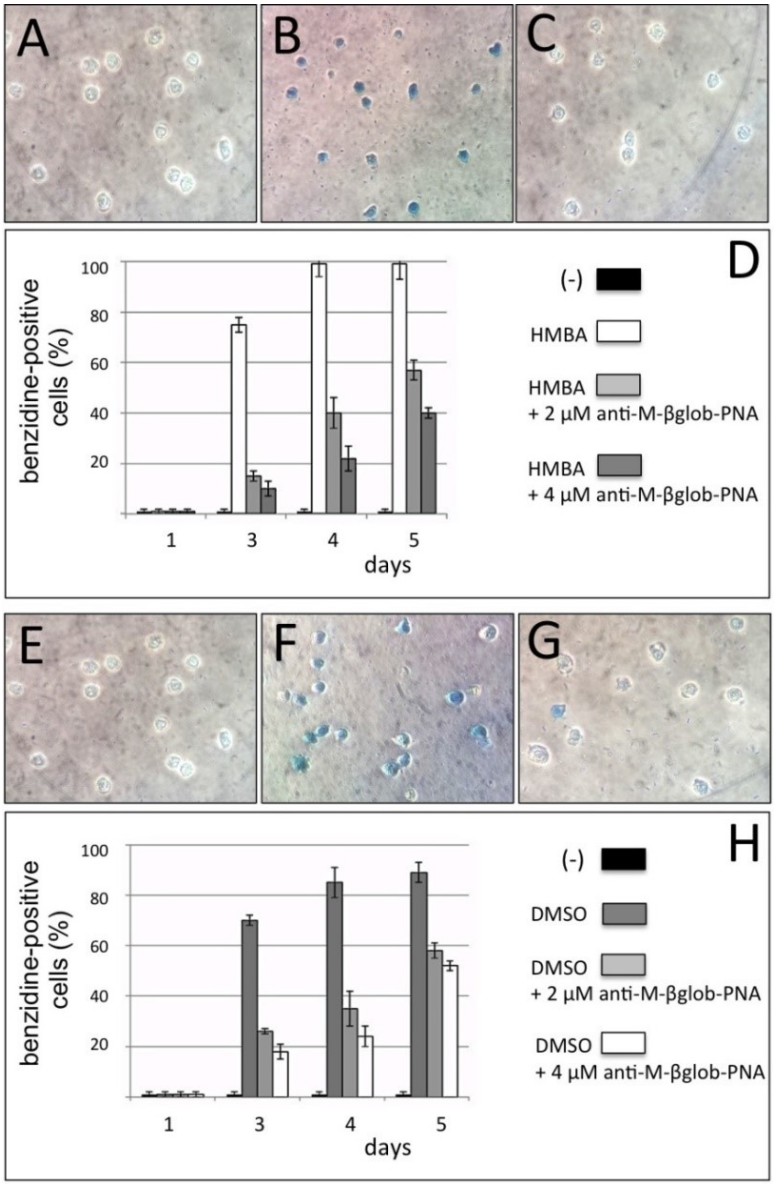


Figure 55. Inibitory effects of anti-M-βglob-PNA on erythroid differentiation of murine erythroleukemia (MEL) cells induced by (A-D) HMBA or (E-H) DMSO. Intracellular accumulation of hemoglobin was determined by the benzidine staining of treated cells. (**A-C** and **E-G**) Representative examples of benzidine staining of (**A** and **E**) untreated cells, or cells treated with (**B**) HMBA, (**C**) HMBA plus 4 µM anti-M-βglob-PNA, (**F**) DMSO or (**G**) DMSO plus 4 µM anti-M-βglob-PNA. (**D-H**) Summary of 3 independent experiments performed with (**D**) HMBA or (**H**) DMSO without the addition of the PNA or in the presence of the indicated concentrations of anti-M-βglob-PNA (From Montagner et al., *Int J Mol Med*, 2015).

3.2.4. Effects of anti-M-βglob-PNA on hemoglobin and β-globin mRNA accumulation in MEL cells treated with HMBA

The hemoglobin production of the induced MEL cells after the treatment with anti-M-βglob-PNA was verified by HPLC analysis of cells lysates. Figure 56 clearly illustrates that anti-M-βglob-PNA inhibited the accumulation of both Hb^{major} and Hb^{minor} hemoglobins fully in agreement with the data shown in Figure 56. Of note, anti-M-βglob-PNA also inhibited, to a certain extent, β-globin mRNA, suggesting that the inhibition of hemoglobin production may be also associated with the lower stability of β-globin mRNA (data not shown).

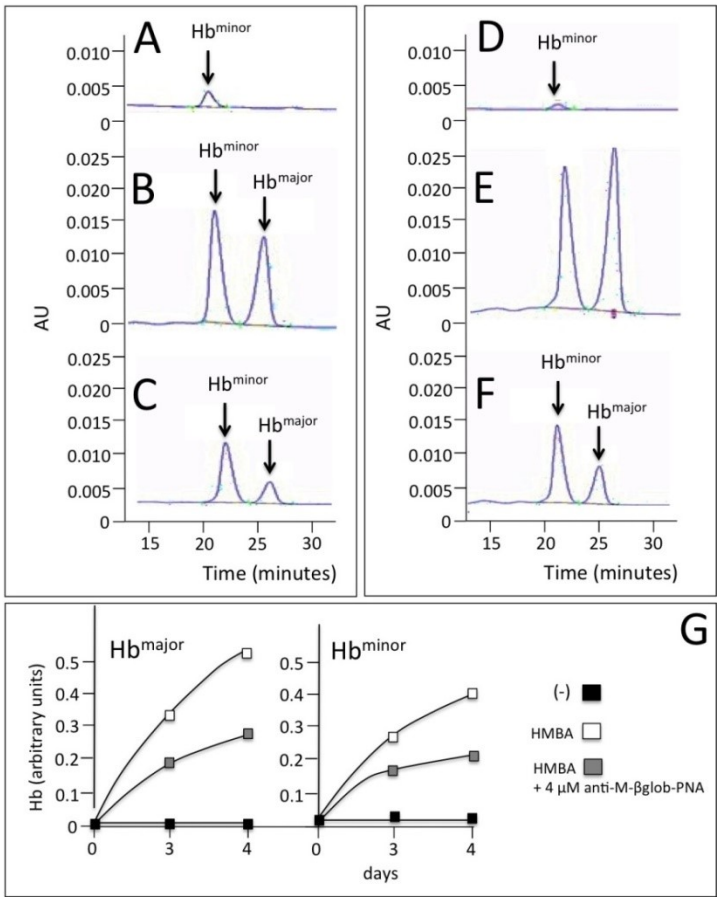


Figure 56. Effects of anti-M-βglob-PNA on hemoglobin accumulation. Representative HPLC analysis of (**A** and **D**) untreated murine erythroleukemia (MEL) cells, (**B** and **E**) hexamethylene bisacetamide (HMBA)-treated cells or (**C** and **F**) cells treated with HMBA in the presence of 4 µM anti-M-βglob-PNA; (**A-C**) 3 days of treatment and (**D-F**) 4 days of treatment. (**G**) Quantitative analyses of Hb^{major} and Hb^{minor} (From Montagner et al., *Int J Mol Med*, 2015).

3.2.5. Lack of effects of anti-M-βglob-PNA on erythroid differentiation of K562-D5 cells induced by Mithramycin (MTH)

The effects of anti-M-βglob-PNA were also examined using human K562 cells subjected to erythroid differentiation by treatment with Mithramycin (MTH). As shown in Figure 57 no inhibitory effects of anti-M-βglob-PNA were observed on the MTH-stimulated K562-D5 cell clones, indicating high levels of specificity of the inhibitory effects of the anti-M-βglob-PNA. Furthermore, control experiments were also performed using the anti-H-γglob-PNA on HMBA- and DMSO-treated MEL cells. Of note, no inhibitory effects were observed using the anti-H-γglob-PNA, suggesting that the effects of treatment of the erythroid cells with PNAs were sequence-specific (Figure 58).

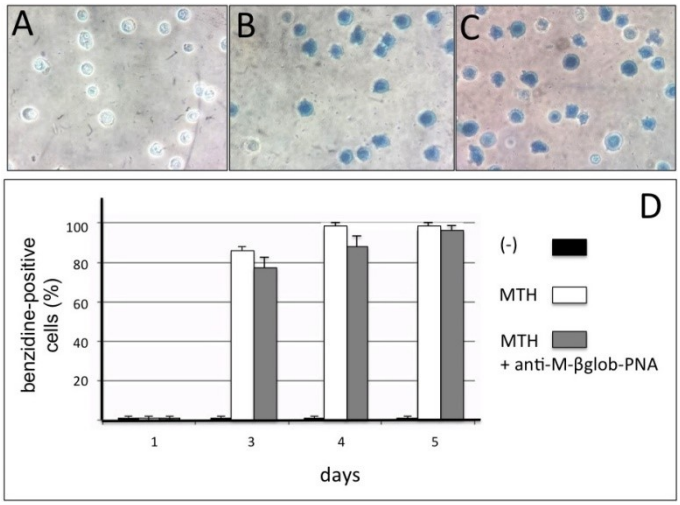


Figure 57. Specificity of the effects of anti-M-βglob-PNA. K562-D5 cells were induced by Mithramycin (MTH). Intracellular accumulation of hemoglobin was determined by benzidine staining. (**A-C**) Representative examples of benzidine staining of (**A**) untreated K562-D5 cells, or cells treated for 4 days with (**B**) MTH or (**C**) MTH plus 4 μM anti-M-βglob-PNA. (**D**) Summary of 3 independent experiments. The proportion of benzidine-positive cells was determined at the beginning of the treatment (day 1) and after 4, 5 and 6 days of cell culture without treatment (black boxes), or treatment with MTH 20 nM in the absence (white box) or in the presence (grey boxes) of 4 μM anti-M-βglob-PNA (From Montagner et al., *Int J Mol Med*, 2015).

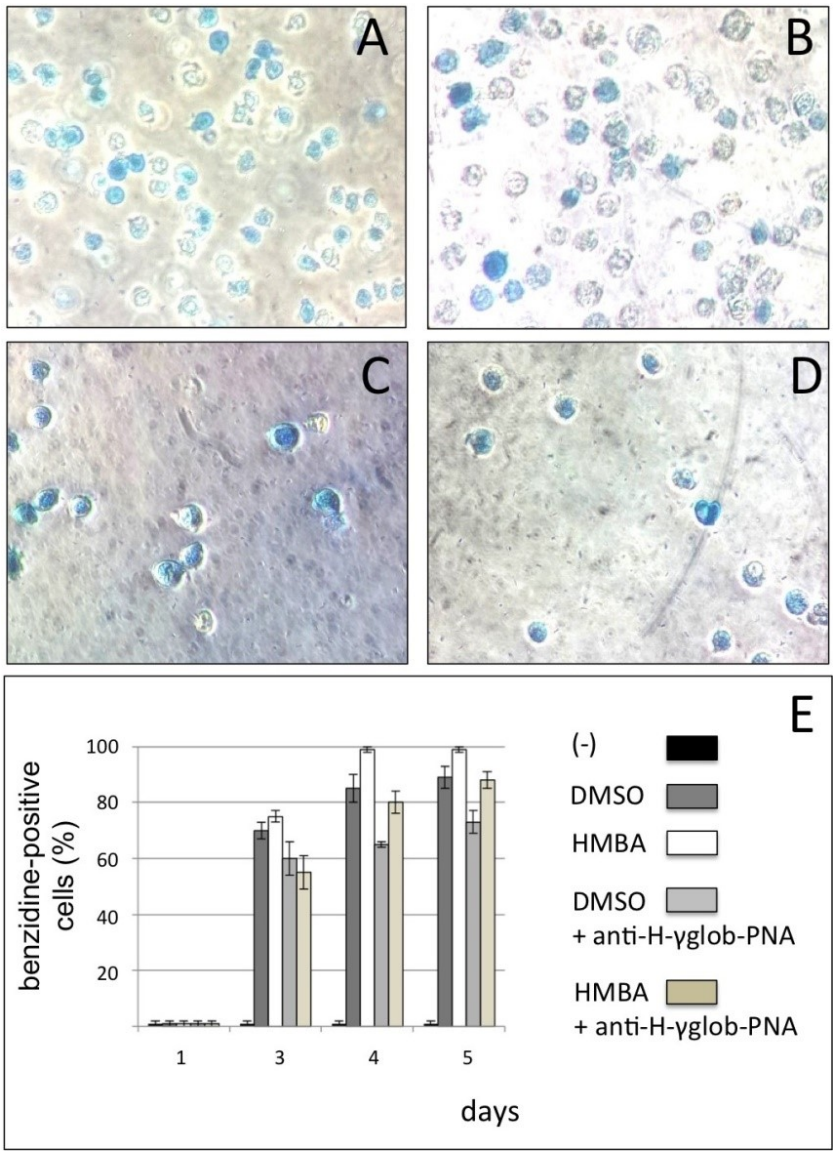


Figure 58. Lack of effects of anti-H-γglob-PNA on erythroid differentiation of murine erythroleukemia (MEL) cells induced by dimethylsulfoxide (DMSO) or hexamethylene bisacetamide (HMBA). (**A-D**) Representative examples of benzidine staining of MEL cells treated for 3 days with (**A** and **B**) 2% DMSO or (**C** and **D**) 2.5 mM HMBA (**A** and **C**) in the absence or (**B** and **D**) in the presence of 4 μM anti-H-γglob-PNA. (**E**) Summary of 3 independent experiments (From Montagner et al., *Int J Mol Med*, 2015).

3.3. DISCUSSION

The buildup of α-chains in β-thalassemia patients initiate an oxidative damage cascade and form damaging precipitates that contribute largely to the clinical problems associated with this disease (Khandros et al., 2012). In sickle cell anemia, the polymerization of HbS is responsible for the sickling of SCA red-blood cells and important adverse clinical parameters (Kaul et al., 1996; Rodgers, 1997). Therefore, the inhibition of accumulation of this defective globin may be beneficial and the antisense properties of peptide nucleic acids might be a potential strategy for this purpose. We have explore this application of PNAs in murine erythroleukemia (MEL) cells, since they express, after treatment with different inducers, almost adult hemoglobin. Two different PNAs were designed, one targeting murine β-globin mRNA and the other targeting human γ-globin mRNA. The anti-M-βglob-PNA exhibited no antiproliferative effects on MEL cell, even in association with the inducers HMBA and DMSO. Benzidine assay demonstrated that MEL cells treated with HMBA or DMSO do not efficiently differentiate after the treatment with anti-M-βglob-PNA. HPLC analysis of cell lysates confirmed the results since the treatment with anti-M-βglob-PNA inhibited the accumulation of both Hb^{major} and Hb^{minor}. In K562-D5 cells induced with Mithramycin the treatment with anti-M-βglob-PNA doesn't reduced the percentage of benzidine-positive cells, demonstrating a specificity of action. Moreover, the treatment with anti-H-γ-globin-PNA has no effects on MEL cells differentiation. Our data suggest that the PNA-based method can inhibit the production of globin chains and may be a therapeutic approach of hemoglobinopathies.

4. ANTISENSE PNA AGAINST SICKLE β-GLOBIN mRNA IN HUMAN ERYTHROLEUKEMIA CELL

In order to evaluate whether a peptide nucleic acid designed to target sickle β-globin mRNA is able to reduce the production of sickle hemoglobin (HbS), erythroid precursors cells from SCD patients have been cultured and treated with the oligonucleotide. After observing the effects on cell growth and differentiation of ErPCs, hemoglobin production has been detected by HPLC analysis.

4.1. MATERIAL AND METHODS

4.1.1. Designed of Anti-βSglob-PNA

Prof. Roberto Corradini and his collaborators (University of Parma, Dept. of Organic and Industrial Chemistry) have synthesized the PNA used in our experiment. The oligonucleotide was linked to an oligoarginine tail R_8 to facilitate intracellular uptake. The sequence of the anti-β^Sglob-PNA is riported in Table 17.

Table 17. Sequence of the peptide nucleic acid used in this experiments.

	SEQUENCE
Anti-β^Sglob-PNA	H-R_8-ACT TCT CCA CAG GAG-Gly-NH_2

4.1.2. Culture of erythroid precursors cell isolated from peripheral blood of sickle cell anemia patients

The culture of human erythroid progenitors were performed as described on page 32 of this PhD thesis. The treatments of ErPCs with anti-β^Sglob-PNA have been performed on the first day of phase II and also on the seventh day. Blood samples were collected from patients Fe75 (beta 6(A3) Glu>Val /beta + IVS I-110) and Fe81 (homozygous beta 6(A3) Glu>Val).

4.1.3. Effects of Anti-β^Sglob-PNA on cell growth and differentiation of erythroid precursors cells

Cell growth analysis, benzidine test and HPLC analysis have been carried out on the twelfth day of phase II of culture. Cell growth was monitored by determining the cell number/ml using the BECKMAN COULTER® Z2 (Beckman, Pasadena, California, USA) in the proper range (6-12 μm for ErPCs). Erythroid differentiated cells were detected by specific reaction with a benzidine/hydrogen peroxide solution (0.2% benzidine in 5mol/l glacial acetic acid, 10% H_2O_2).

4.1.4. High performance liquid chromatography (HPLC) analysis

The cells were harvested, washed once with PBS and the pellets were lysed in lysis buffer (sodium dodecyl sulphate 0.01%). After spinning for 1 min in a microcentrifuge, the supernatant was collected and stored at 4˚C. Hemoglobins in the lysates were separated by cation-exchange HPLC (Pharmacia LKB Gradient Pump 2249, VWM 2141), using a Synchropak CM300 (250x4.6 mm) column (Eichrom Technologies, Inc., Darien, IL, USA) and BisTris (30 mM) buffer.

4.2. RESULTS

4.2.1. Effects of the anti-β^Sglob-PNA on the growth of erythroid precursors cells and differentiation

The growth of erythroid precursors cells treated with different concentration of anti-β^Sglob-PNA is depicted in Figure 59A, where it is shown that no important antiproliferative effect of the oligonucleotide occurs in human erythroid progenitors. The differentiation of the cells and thus the production of hemoglobin was lower than untreated cells, as represented in Figure 59B.

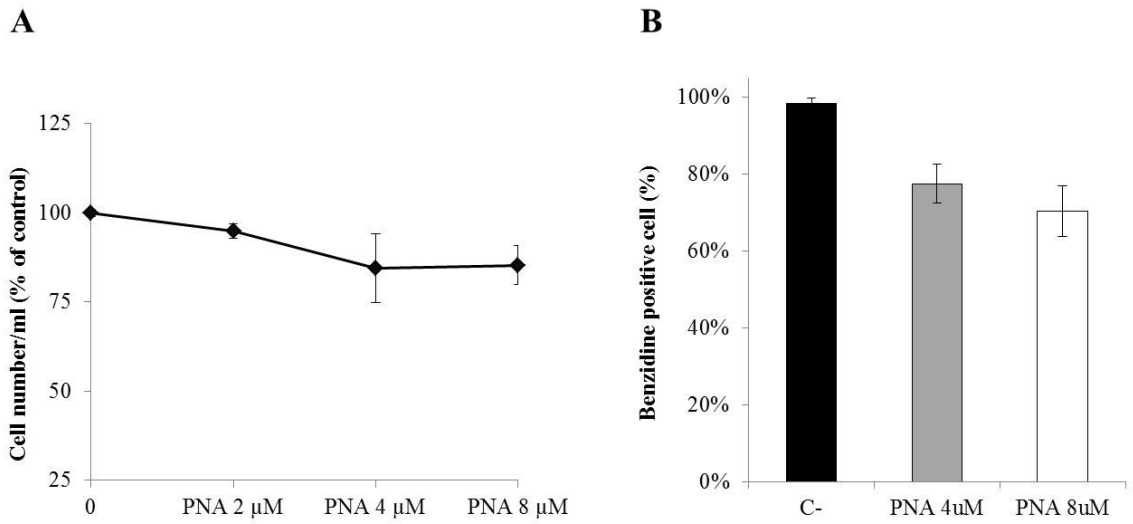


Figure 59. Cell growth (A) and differentiation (B) of erythoid precursors treated with anti-β^Sglob-PNA. Erythroid precursors cells have been isolated from peripheral blood of SCD patients. Anti-β^Sglob-PNA exhibited no important antiproliferative effect on cell growth (**A**) but was able to reduce the production of hemoglobin (**B**). The PNA reduces the percentage of benzidine-positive cells up to 30%.

4.2.2. Effects of anti-β^Sglob-PNA on hemoglobin accumulation in erythroid precursors cells

The hemoglobin production of ErPCs after the treatment with anti-β^Sglob-PNA was verified by HPLC analysis of cells lysates. Figure 60 reports the inhibition of hemoglobin accumulation in erythroid precursors cell of patient Fe75 (A) and Fe81 (B) treated with 4 micromolar PNA. It is clearly demonstrated that the administration of anti-β^Sglob-PNA reduces also the amount of both HbF, HbA_2 but preferentially of sickle hemoglobin (HbS), especially in patient ErPCs from Fe81.

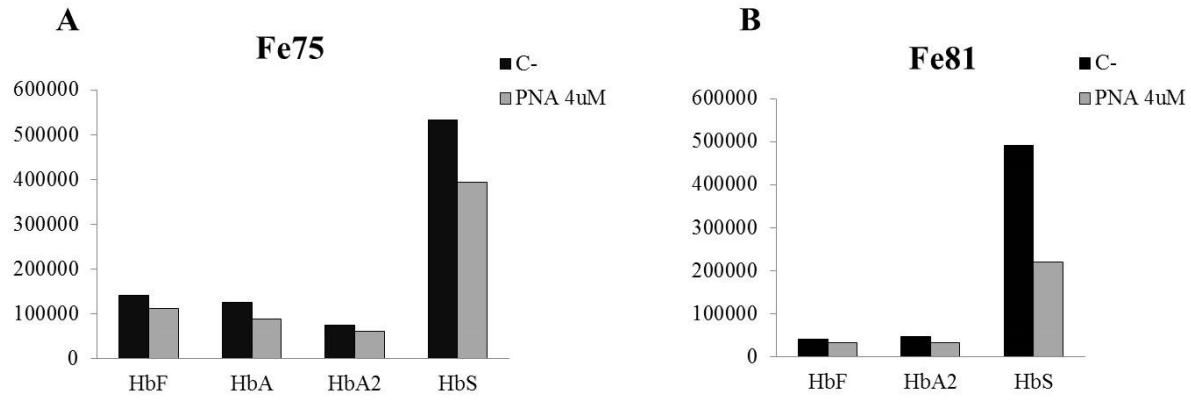


Figure 60. Accumulation of hemoglobin in erythroid precursors cells treated with 4 micromolar anti-β^Sglob-PNA. The amount of fetal hemoglobin (HbF) adult hemoglobin (HbA and HbA_2) and sickle hemoglobin (HbS) in cell lysates are represented as c*hromatographic peak integration* obtained by HPLC analysis.

Moreover, we have performed another treatment with anti-β^Sglob-PNA 8 μM in erythroid precursors cells from patient Fe75. The result indicates that a reduced hemoglobin accumulation occurs after the treatment with the oligonucleotide. As reported in Figure 61, even if the reduction of hemoglobin accumulation are not restricted to HbS, the antisense effect of anti-β^Sglob-PNA is conspicuous mostly against sickle hemoglobin.

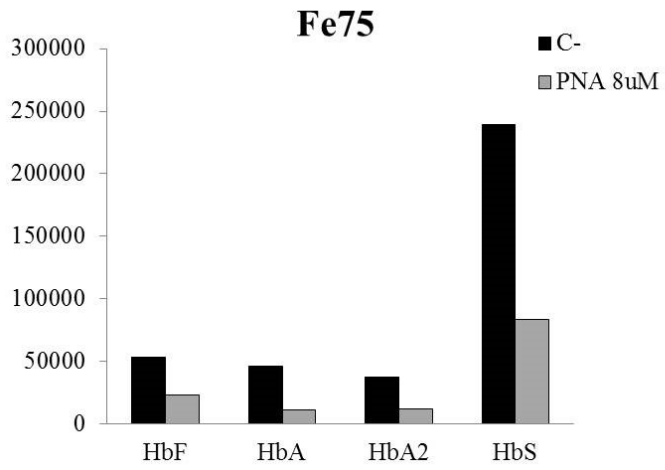


Figure 61. Accumulation of hemoglobin in erythroid precursors cells treated with 8 micromolar anti-β^Sglob-PNA. The amount of fetal hemoglobin (HbF) adult hemoglobin (HbA and HbA_2) and sickle hemoglobin (HbS) in cell lysates are represented as c*hromatographic peak integration* obtained by HPLC analysis. The reduction affects HbF, HbA and HbA_2 but preferentially HbS, also in this case.

4.3. DISCUSSION

The polymerization of sickle hemoglobin in SCD is responsible for the characteristic sickle shape and the reduced lifespan of red blood cells. Therefore, HbS appears to be an important therapeutic target. Since in our previous study we have demonstrated the antisense activity of a peptide nucleic acid against murine β-globin mRNA (Montagner et al., 2015), a PNA targeting sickle β-globin has been designed by Prof. Roberto Corradini (University of Parma, Dept. of Organic and Industrial Chemistry) and his collaborators.

The anti-β^Sglob-PNA was tested in erythroid precursors cells isolated from SCD patients and cultured following the two-phase liquid culture procedure. The PNA displayed no relevant antiproliferative effects but the percentage of benzidine-positive cells was reduced with respect to untreated cells, reflecting the lower accumulation of hemoglobins. This data was in agreement with the results obtained by HPLC analysis. In fact, after the treatment of ErPCs from patients Fe75 and Fe81, the accumulation of hemoglobins was found to be decreased. The effect of anti-β^Sglob-PNA was not restricted to HbS but nevertheless the antisense activity was preferential on the sickle hemoglobin. These findings were confirmed by the results obtained after the treatment of ErPCs from patient Fe51 with anti-β^Sglob-PNA at higher concentration.

In conclusion, since our results demonstrate the ability of a PNA to reduce the accumulation of sickle hemoglobin, we propose further investigation on this antisense strategy for the therapy of SCD in order to ameliorate the selectivity of the oligonucleotide employed.

DISCUSSION AND GENERAL CONCLUSIONS

Among the hemoglobinopathies, sickle cell disease (SCD) and β-thalassemia have the most impact on morbidity and mortality, affecting millions worldwide (Weatherall, 2010). Around 1-2% of the global population are heterozygous for HbS and 3% are heterozygous for β-thalassemia (Theodorsson et al., 2007). Both are prototypical Mendelian single gene disorders affecting the β-globin gene. Despite the apparent genetic simplicity, both disorders display extreme clinical heterogeneity (Steinberg et al., 2009; Galanello et al., 2010). Sickle cell anemia and β-thalassemia patients with an HPFH (Hereditary Persistence of Fetal Hemoglobin) phenotype display milder clinical parameters, since increased levels of fetal hemoglobin (HbF, $\alpha 2\gamma 2$) confer major clinical benefits. Therefore reactivation of γ-globin gene expression and HbF production is one of the best strategies to ameliorate the symptoms of patients with β-hemoglobinopaties. Hydroxyurea (HU), the only fetal hemoglobin inducer approved by the US Food and Drug Administration, is currently the only established preventative pharmacologic against painful crises in SCD. Moreover, several independent *in vivo* trials demonstrated that the treatment of β-thalassemia patients with the Hydroxyurea leads to a clear improvement of the blood-intake requirements and even transfusion-independency in about 40-60% of the cases (Italia et al., 2009; Ansari et al., 2011). Despite this, the identification of novel HbF inducers with higher efficiency lower toxicity in comparison with HU must be carried on in order to ensure a personalized therapy of β-hemoglobinopaties.

Sirolimus (or Rapamycin) was largely described as a fetal hemoglobin inducers in human erythroleukemia cells and erythroid precursors cells by several studies (Yamamoto-Yamaguchi et al., 2001; Mischiati et al., 2004; Fibach et al., 2006; Pecoraro et al., 2015). Rapamycin is an attractive molecule since its effect is not associated with cytotoxicity and cell growth inhibition and interestingly, the dosages that was found effective *in vitro* were very similar to those described to be present in the blood of kidney transplanted patients treated with Rapamycin (Gummert et al., 1999). In addition, a remarkable study brought out that Sirolimus reduced the rate of red blood destruction, decreased the transfusion rate and caused a reduction in iron deposition after an *in vivo* treatment of a patient with β-thalassemia major (Rak et al., 2013). Therefore, further experiments were necessary in order to validate the preliminary results obtained in erythroid precursors cells and to take in consideration Rapamycin as a therapeutic molecule for β-thalassemia and sickle cells anemia. Erythroid precursors cells (ErPCs) from peripheral blood were isolated from 33

patients with 14 different genotypes. ErPCs from high HbF expressing patients were found to be responsive to Sirolimus with higher efficiency with respect to ErPCs from low HbF expressing patients. A comparison of the responses of ErPCs to Hydroxyurea treatment and Rapamycin treatment revealed that in some cases, ErPCs are preferentially induced by Sirolimus. The induction of HbF was remarkable also in Hydroxyurea-resistant erythroid cells. The transcriptomic profile of treated ErPCs indicated that HU and Sirolimus induce differential effects on the overall gene expression, possibly reflecting a different mechanism of action. Hence, we performed the combined treatment of ErPCs with the two inducers. The data obtained revealed that the co-administration of Sirolimus plus HU were more efficient than HU in a subset of patients in inducing HbF. For all these findings, we believe that Rapamycin should be taken into account for a clinical trial aimed at identifing the best fetal hemoglobin inducer with the view to establish a personalized therapy of β-thalassemia and sickle cell anemia.

Resveratrol, a natural polyphenolic compounds was found to induce differentiation of K562 cells and to augment fetal hemoglobin (HbF) production in K562 cells and in erythroid precursors isolated from SCA patients (Rodrigue et al., 2001). Another study reports that the phytoalexin increased fetal hemoglobin as well as γ-globin mRNA significantly and reproducibly in cultures of erythroid progenitors of β-thalassemia patients (Fibach et al., 2012). The interest on Resveratrol in the therapy of β-thalassemia is also supported by its antioxidant activity; in fact, oxidative stress has an important role in β-hemoglobinopaties, since red blood cells, as well as platelets and polymorphonuclear leukocytes (PMN) derived from such patients, are under oxidative stress (Amer et al., 2006). No adverse effects of Resveratrol have been described until now, hence our research focused on the activity of Resveratrol-nutraceutical in erythroid precursors cells. In preliminary studies, the nutraceutical product Transmax® (Biotivia) exhibited the greater *in vitro* induction of γ-globin transcripts and of fetal hemoglobin in ErPCs from patients with β-thalassemia. Transmax® was administered to patients at a concentration equal to that suggested in normal subjects, who take the nutraceutical product for its antioxidant activity. The study was carried out at the Pediatric Oncohematology Unit, Santa Chiara Hospital (Pisa) in collaboration with Dott. Claudio Favre. Four β-thalassemia patients have been taken into consideration in this PhD thesis. Resveratrol induction of γ-globin mRNA was evident during the treatment of the four β-thalassemia patients. The relative expression of γ-globin in relation to α-globin fold mRNA after one year, when patients have not taken Resveratrol-nutraceutical, was found to be significantly lower.

Moreover, the production of fetal hemoglobin analyzed by HPLC was increased during the treatment but also during follow-up. Thus, *in vivo* administration of Resveratrol-nutraceutical might be repurposed into a wider number of patients with β-thalassemia but also sickle cell anemia, with more detailed clinical analysis and with further assessment of the production of fetal hemoglobin in reticulocytes.

Angelicin is an angular furanocoumarin naturally present in the fruit or root of *Angelica Archangelica* (Apiacee). It has been demonstrated that this compound can increase the content of fetal hemoglobin in erythroid progenitors from normal subjects and the amount of γ-globin transcript in erythroid precursor cells isolated from β-thalassemia patient (Lampronti et al., 2003). In order to reduce undesirable side effects, especially long-term ones, such as genotoxicity, new analogues of Angelicin have been synthetized by Prof. Adriana Chilin (Department of Pharmaceutical and Pharmacological Sciences of Padua, Italy). The 38 compounds were tested in human erythroleukemia cell line K562 and four of them, were found to be able to induce the erythroid differentiation of more than 15% of cells. This four molecules (GMB-06, GMB-08, GMB-21, GMB-26) were tested in ErPCs from β-thalassemia patients and were found to be able to induce γ-globin gene expression and fetal hemoglobin production at different level, without relevant antiproliferative effects. The results indicate that further experiments in a larger number of blood samples from β-thalassemia patients in order to identify the most effective compound.

In β-thalassemia unbalanced production of α- and β-globin chain has an even more profound impact upon clinical severity. Unpaired insoluble α-globin chains accumulate and precipitate and the degradation of α- monomers elicit redox reaction (Shaeffer et al., 1988). Ineffective erythropoiesis and death of bone marrow erythroid precursors appear to be a result of the mechanism triggered by reactive oxygen species. In sickle cell anemia, the polymerization of sickle hemoglobin (HbS) deforms the shape of red blood cells, which have consequently a reduced lifespan. Therefore, drug-based methods enabling efficient inhibition of accumulation of this defective globin can be of great interest. Peptide nucleic acids as antisense molecules targeting mRNAs have been employed in this field of investigation.

We have at first test an innovative delivery system of PNA in erythroid cells based on a liposomal formulation developed by Prof. Alessandra Romanelli (Department of Pharmacy, University of Naples). FACS analysis revealed a fast uptake of the liposomes carrying the PNA against miRNA-210 in K562 cell lines and the nuclei staining

highlighted a mostly cytoplasmic localization of PNA, confirmed by microscopic analysis. The liposomal formulation caused only minor effects on the rate of cell growth and the expression of miRNA-210 was reduced after the treatment with LIPO-PNA-a210 for 48h. Moreover, it has been demonstrated that the liposome-delivered PNA-a210 maintained its specificity on miR-210 downregulation, since the expression of the unrelated miR-221 were not affected by LIPO-PNA-a210 (Avitabile et al., 2015).

A PNA targeting murine adult β-globin mRNAs (anti-M-βglob-PNA) and another recognizing the human γ-globin mRNAs (anti-H-γ-globin-PNA) were produced by Prof. Roberto Corradini (University of Parma, Dept. of Organic and Industrial Chemistry) in order to verify whether this strategy can be used to modulate globin gene expression and to reduce the level of a given type of globin . Murine Erythroleukemia (MEL) cells were chosen as experimental model system since they express, after treatment with different inducers, almost adult hemoglobin. No antiproliferative effects of the anti-M-βglob-PNA were observed on MEL cell, even in association with the inducers HMBA and DMSO. The anti-M-βglob-PNA reduced the percentage of differentiated MEL cells treated with HMBA or DMSO and inhibited the accumulation of both Hb^{major} and Hb^{minor}. The effects was selective since in K562-D5 cells induced with Mithramycin the treatment with anti-M-βglob-PNA doesn't reduced the percentage of benzidine-positive cells. Moreover, the treatment with anti-H-γ-globin-PNA has no effects on MEL cells differentiation (Montagner et al., 2015). Our study allows to propose PNAs to down-regulate hemoglobins involved in the onset of pathological conditions such as SCD and β-thalassemia.

In this field of investigation, erythroid precursors cells from SCD have been treated with a peptide nucleic acid targeting sickle β-globin mRNA (anti-β^Sglob-PNA). The accumulation of hemoglobin was decreased after the treatment with anti-β^Sglob-PNA without relevant antiproliferative effects; HPLC analysis of cell lysates revealed that the reduction of hemoglobin accumulation were not restricted to HbS, but the antisense effect of anti-β^Sglob-PNA were remarkable mostly against sickle hemoglobin. Hence, further investigation are necessary to enhance the specificity of the peptide nucleic acid against sickle β-globin mRNA. Although, taken together, these results indicate that the PNA-based strategy could have an important role as an innovative strategy for the treatment of β-hemoglobinopathies.

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