Universidade de Aveiro Departamento de Biologia
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André Filipe Gonçalves Gabriel

Terapia de supressão da β-talassemia usando Canamicina e Gentamicina

Suppression therapy of β-thalassemia using Kanamycin and Gentamicin

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Luís Manuel Souto de Miranda, Professor Auxiliar Convidado do Departamento de Biologia da Universidade de Aveiro e da Doutora Luísa Maria Ferreira Romão Loison, Investigadora Principal do Departamento de Genética Humana do Instituto Nacional de Saúde Dr. Ricardo Jorge.

I don’t want to believe. I want to know.

Carl Sagan

o júri

presidente

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agradecimentos

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palavras-chave

β-talassemia, β-globina, NMD, PTC, canamicina, gentamicina, terapia de supressão.

resumo

As mutações *nonsense* são mutações pontuais que originam codões de terminação prematura (PTCs). A expressão de genes portadores de PTCs pode levar à síntese de proteínas truncadas. As proteínas truncadas caracterizam-se por serem menores e, na maioria das vezes, não possuem função biológica, apesar de poderem ter funções deletérias para a célula. Em condições normais, transcritos portadores de PTCs são degradados rapidamente através do processo de *nonsense mediated mRNA decay* (NMD).
Quando um PTC atinge o sítio A ribossomal, os fatores de terminação da tradução ligam-se ao mesmo e a tradução termina imediatamente. A terapia de supressão consiste numa abordagem terapêutica que tem o objetivo de utilizar compostos de baixo peso molecular para induzir a incorporação de aminoacil-tRNAs quase cognatos, moléculas que possuem complementaridade para dois dos três nucleótidos de um códão de stop, quando o ribossoma atinge um PTC. Assim, a tradução não termina prematuramente.
Estudos anteriores mostraram que alguns aminoglicósidos possuem a capacidade de suprimir PTCs responsáveis por doenças, como fibrose quística e distrofia muscular de Duchenne. Algumas mutações *nonsense* são responsáveis pela β-talassemia.
Neste estudo foram utilizados dois aminoglicósidos, canamicina e gentamicina, de modo a avaliar a sua capacidade em aumentar a competitividade de tRNAs quase cognatos com os fatores de terminação da tradução pelo sítio A ribossomal, na presença de um PTC, evitando dessa forma a terminação prematura da tradução.

keywords

β-thalassemia, β-globin, NMD, PTC, kanamycin, gentamicin, suppression therapy.

abstract

Nonsense mutations are point mutations that originate premature termination codons (PTCs). The expression of PTC-containing genes may lead to the synthesis of truncated proteins. Truncated proteins are shorter proteins that at most times do not have biological function, but may have deleterious functions for the cell. In regular conditions, PTC-containing transcripts are taken to rapid decay, through nonsense mediated mRNA decay (NMD).

When a PTC reaches the ribosomal A-site, translation release factors bind it and translation immediately stops. Suppression therapy is a therapeutic approach that aims to suppress PTCs by using low molecular weight compounds to induce the incorporation of near cognate aminoacyl tRNAs, molecules that show complementarity to two of the three nucleotides of a stop codon, when the ribosome reaches a PTC. Thus, translation does not prematurely terminates.

Previous studies have shown that some aminoglycosides have the ability to suppress PTCs responsible for diseases like cystic fibrosis and Duchenne muscular dystrophy. Some nonsense mutations are responsible for β-thalassemia disease.

In this study two aminoglycoside compounds, kanamycin and gentamicin, were used in order to evaluate their capacity to increase the competition of near cognate aminoacyl tRNAs with translation release factors by the ribosomal A-site, when the ribosome reaches a PTC, therefore avoiding the premature termination of translation.

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ºC – Degrees Celsius

A – Adenine

APS – Ammonium persulfate

AS – Alternative splicing

A-site – Aminoacyl-site

bp – Base pairs

C – Cytosine

CBP80 – Nuclear cap-binding protein subunit 1

cDNA – Complementary deoxyribonucleic acid

CFTR – Cystic fibrosis transmembrane conductance regulator

CO_2 – Carbon dioxide

C-terminal – Carboxyl-terminal

DECID – Decay inducing complex

DMD – Duchenne muscular dystrophy

DMEM – Dulbecco's modified Eagle medium

DNA – Deoxyribonucleic acid

dNTP - Deoxyribonucleotide triphosphate

DTT - Dithiothreitol

eEF – Eukaryotic translation elongation factor

eIF – Eukaryotic translation initiation factor

eiF4AIII – Eukaryotic translation initiation factor 4A3

EJC – Exon junction complex

eRF – Eukaryotic release factor

E-site – Exit-site

FBS – Fetal bovine serum

G – Guanine

GDP – Guanosine diphosphate

GTP – Guanosine triphosphate

H_2O – water

HbA – Adult hemoglobin

HbA2 – Adult hemoglobin 2

HbF – Fetal hemoglobin

HCl – Hydrochloric acid

HeLa – human cervical cancer cell line

HRP – Horseradish peroxidase

IgG - Immunoglobulin G

kDa – Kilodalton

LB – Luria Bertani medium

M - Molar

m7G – 7-methylguanosine

mA - Milliamp

Magoh – Mago nashi homolog

MEL – Murine erythroleukemia cell line

MEM – Minimal essential medium

Met – Methionine

Met-$tRNA_i^{Met}$ – Methionyl-initiator tRNA

$MgCl_2$ – Magnesium chloride

mL – Milliliter

mM – Millimolar

mm – Millimeter

mRNA – Messenger ribonucleic acid

ng – Nanograms

NMD – Nonsense mediated mRNA decay

NT – Non transfected

N-terminal – Amino-terminal

ORF – Open reading frame

P – Phosphate

PABP – Polyadenylate-binding protein

PABPC1 – Cytoplasmic polyadenylate-binding protein

PAGE - Polyacrylamide gel electrophoresis

P-bodies – Processing bodies

PBS - Phosphate-buffered saline

PCR – Polymerase chain reaction

pH – Power of hydrogen

PIC – Preinitiation complex

PIN – Pil-T N-Terminus

pmol – Picomol

PNRC2 – Proline-rich nuclear receptor coactivator 2

Poly(A) – Polyadenylate

PP2A – Phosphatase 2A

P-site – Peptidyl-site

PTC – Premature termination codon

$Puro^R$ – Puromycin resistance gene

PVDF – Polyvinylidene difluoride

RNA – Ribonucleic acid

RNP – Ribonucleoprotein

rpm – Rotations per minute

RT – Reverse transcription

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

S – Svedberg

SB – Sample buffer

SDS – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS-PAGE – Sodium dodecyl sulfate

SMG – UPF1 kinase suppressor with morphogenetic effect on Genitalia 1

SURF – SMG1-UPF1-eRF1/3 complex

T – Thymine

TBS – Tris-buffered saline

TEMED – Tetramethylethylenediamine

tRNA – Transfer ribonucleic acid

U – Uracil

uORF – Upstream open reading frame

UPF – Up-frameshift

UTR – Untranslated region

V – Volt

v/v – Volume/volume

w/v – Weight/volume

XRN1 – 5'-3' exoribonuclease 1

β15 – Human β-globin mRNA containing a PTC at codon 15

β39 – Human β-globin mRNA containing a PTC at codon 39

β127 – Human β-globin mRNA containing a PTC at codon 127

βN – Wild type human β-globin mRNA

µg – Microgram

µL – Microliter

µM – Micromolar

1. Literature review

1.1. Beta-thalassemia

β-thalassemia consists of a heterogeneous group of diseases caused by mutations in the β-globin gene (HBB), present in the human chromosome 11, which lead to a reduced production of β-globin protein (Rund *et al*., 2005). It is estimated that about 1.5% of the world population carries β-thalassemia and that about 60000 individuals were born with β-thalassemia symptoms (Galanello and Origa, 2010).

HbA (adult hemoglobin) consists of four globin chains: two α-chains and two β-chains. In a regular situation, the HbF (fetal hemoglobin) γ-chains are completely replaced by β-chains during the first six weeks after birth. In a β-thalassemia case, the symptoms of the disease start to arise just a few months after birth, during the time when γ-chains replacement should occur. This happens because there are no β-chains to be substituted (Galanello *et al*., 1989; Cao *et al*., 2010). The reduced production of β-globin leads to the precipitation of α-chains within and on the membrane of erythrocytes, resulting in the formation of Heinz bodies. The presence of free α-globin chains in the bone marrow, leads to their precipitation in erythrocytes leading to membrane disruption and consequent reduction in erythropoiesis efficiency. Therefore, there is a reduction in the number of erythrocytes in the bloodstream (Steinberg *et al*., 2001; Cao *et al*., 2010).

Three types of β-thalassemia can be characterized based on the clinical manifestations: β-thalassemia major, with more severe symptoms such as jaundice, splenomegaly, typical skeletal variants with stunted growth and reduction in the average life expectancy (Borgna-Pignatti *et al*., 2004); β-thalassemia intermedia, being less intense and having less need for therapeutic intervention (Wainscoat *et al*., 1987, Galanello *et al*., 1989; Ho *et al*., 1998); β-thalassemia minor, in which individuals carry a single

mutant allele, and do not show symptoms, despite the possible occurrence of microcytic and hypochromic erythrocytes. Individuals with β-thalassemia minor have a higher concentration of HbA2 in the bloodstream (Steinberg *et al*., 2001; Borgna-Pignatti *et al*., 2004).

1.2. Messenger RNA translation

Protein synthesis occurs in the cell cytoplasm and is directly dependent on the translation machinery, constituted by the ribosome, tRNA molecules, tRNA aminoacyl synthetases, initiation factors and the mRNA molecule. The translation process consists of four stages: initiation, elongation, termination and recycling. The mRNA molecule provides the information that is read by the translation machinery (Pestova *et al*., 2001; Preiss and Hentze, 2003).

Initiation is the most complex phase of the translation process. The eukaryotic translation initiation factor 3 (eIF3) binds the E-site in the small (40S) ribosomal subunit, in order to prevent the 60S complex from binding it, at the very beginning of the translation process, and in order to allow the 40S subunit to bind the mRNA molecule. eIF5, a GTPase activating enzyme and eIF1 and eIF1A also bind the 40S subunit. In this stage, it is very important to ensure that only the Peptidyl-site (P-site) is free in order to accept the methionyl-initiator tRNA (Met-$tRNA_i^{Met}$). The eIF2 is a key factor in the translation initiation process. This initiation factor is a GTPase composed by 3 subunits. It carries the Met-$tRNA_i^{Met}$, and brings it to the ribosomal P-site, forming the 43S pre-initiation complex (shortened as 43S PIC). It is noticeable that, once attached to the 43S PIC, the tRNA is still not attached to the mRNA by its anticodon.

(Lamphear *et al*., 1995; Preiss and Hentze, 2003; Agirrezabala and Frank, 2010; Nanda *et al*., 2013).

The eIF4A, eIF4E and eIF4G combine together into the eIF4F complex. eIF4B stimulates the eIF4A activation. It has been shown that eIF4E binds the 5' cap. The interaction between the PABPC1, located in the mRNA's 3' poly(A) tail, and the eIF4E with the 5' cap, folds the mRNA into a closed-loop structure which leads to its activation, as shown in the figure 1 (Wells *et al*., 1998; Asano *et al*., 2001; Svitkin *et al*., 2001; Preiss and Hentze, 2003; Yamamoto *et al*., 2005; Nanda *et al*., 2013).

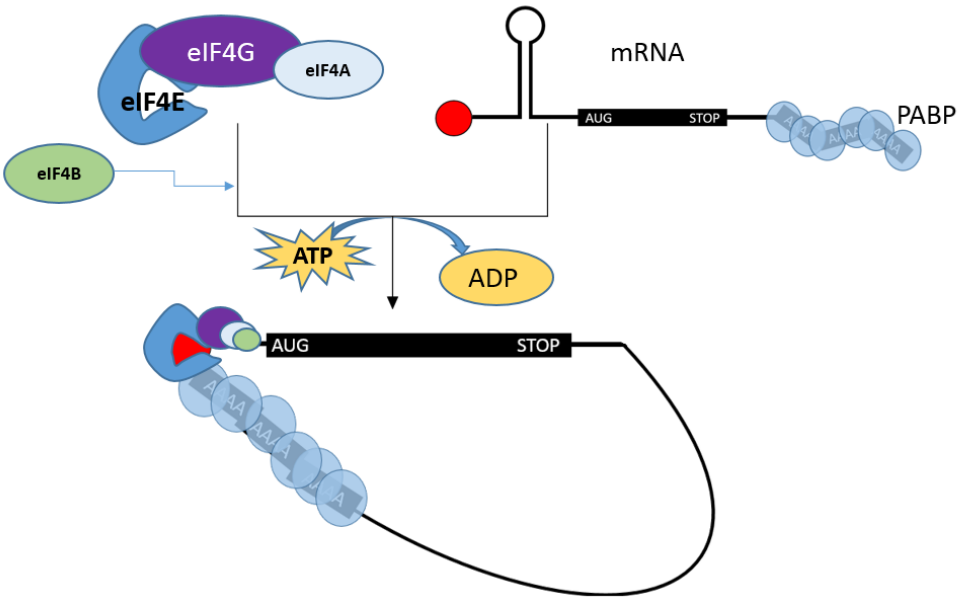


Figure 1: mRNA activation. eIF4E, eIF4G and eIF4A form the eIF4F complex. eIF4B binds to the eIF4A protein. eIF4E binds to the 5' cap. The mRNA molecule folds into a loop-like structure due to the interaction between the PABP, located in the 3' poly(A)tail, and the eIF4E with the 5' cap. This process is energetically expensive because it requires hydrolysis of ATP into ADP and Pi in order to occur. Adapted from Jackson *et al*., 2010.

Scanning of the AUG codon is performed in a 5'-to-3' sense. Once the translation machinery reaches the start codon, eIF2 hydrolyzes GTP into GDP and Pi and the Met-$tRNA_i^{Met}$ binds the AUG codon in the P-site which forms the 48S initiation complex.

The Aminoacyl-site (A-site) is left empty (Svitkin *et al*., 2001; Gebauer *et al*., 2004; Matsuda *et al*., 2006; Jackson *et al*., 2010; Shin *et al*., 2011).

Once the anticodon-codon bond has been established, the large ribosomal subunit (60S) is carried by the GTPase protein eIF5B, and is assembled in the ribosome 40S subunit. As this process occurs, eIF5B hydrolyzes GTP into GDP and Pi and all the other initiation factors are released. Thus, the 80S subunit is assembled and is ready to proceed to the elongation step (Ramakrishnan, 2002; Preiss and Hentze, 2003; Gebauer *et al*., 2004; Jackson *et al*., 2010; Nanda *et al*., 2013).

The elongation step has two major phases, being these the peptide bond formation and the translocation of the ribosome trough the mRNA molecule. The elongation factor 1A (eEF1A) is a GTPase that attaches to the aminoacyl tRNA and brings it to the ribosome A-site, which is empty. This factor is also carrying a GTP molecule. Once in the ribosome A-site, eEF1A hydrolyzes its GTP into GDP and Pi and releases the tRNA which attaches the A-site. The protein eEF1A is recycled by a GTP exchange factor (GEF) composed by the elongation factors 1B, 1D and 1G. The GEF will replace the eEF1A's GDP with GTP, thus eEF1A will be ready to attach another aminoacyl tRNA (Hotokezaka *et al*., 2002; Ramakrishnan, 2002; Gebauer *et al*., 2004; Agirrezabala and Frank, 2010; Li *et al*., 2013).

The peptidyl transferase enzyme has a crucial role in the translation elongation because it catalyzes the formation of a peptide bond between the amino acids located in their respective tRNA molecules in the P- and A-site, respectively. As a consequence, the tRNA present in the P-site releases its amino acid. Then, the ribosome moves to the next codon, by a process called translocation, which requires eEFG and GTP. The tRNA present in the P-site moves to the ribosome Exit-site (E-site) and then leaves the translation machinery. The tRNA that was present in the ribosome A-site is now in the

P-site and, once the A-site is now empty, another aminoacyl tRNA binds to it. Thus, this process is repeated until the ribosome reaches the stop codon (being UAA, UAG or UGA) (Gebauer *et al*., 2004; Agirrezabala and Frank, 2010; Zhou *et al*., 2014).

There are no aminoacyl tRNA molecules that have an anticodon that can pair with any of the three existing stop codons. When the stop codon of the mRNA enters the ribosome A-site, the eukaryotic release factor 1 (eRF1), also known as TB3-1 in humans, and the eukaryotic release factor 3 (eRF3), a GTPase, are recruited (Janzen and Geballe, 2004; Kashima *et al*., 2006). The eRF1 mimics the tRNA in the A-site of the ribosome, recognizing the stop codon. It has a major role in the translation termination, because it cleaves the peptidyl-tRNA bond by hydrolysis. A GTP is associated with the eRF3. This GTP molecule is hydrolyzed into GDP and Pi when the eRF1 binds the A-site, providing sufficient energy which allows the cleavage of the nascent peptide (Frolova *et al*., 1996 and Wang *et al*., 2001; Janzen and Geballe, 2004; Kashima *et al*., 2006). Thus, every translation machinery element is released and recycled as shown in figure 2. The same mRNA molecule may be translated more than once at a time and may also be translated again, before it is taken to its decay (Dever and Green, 2012).

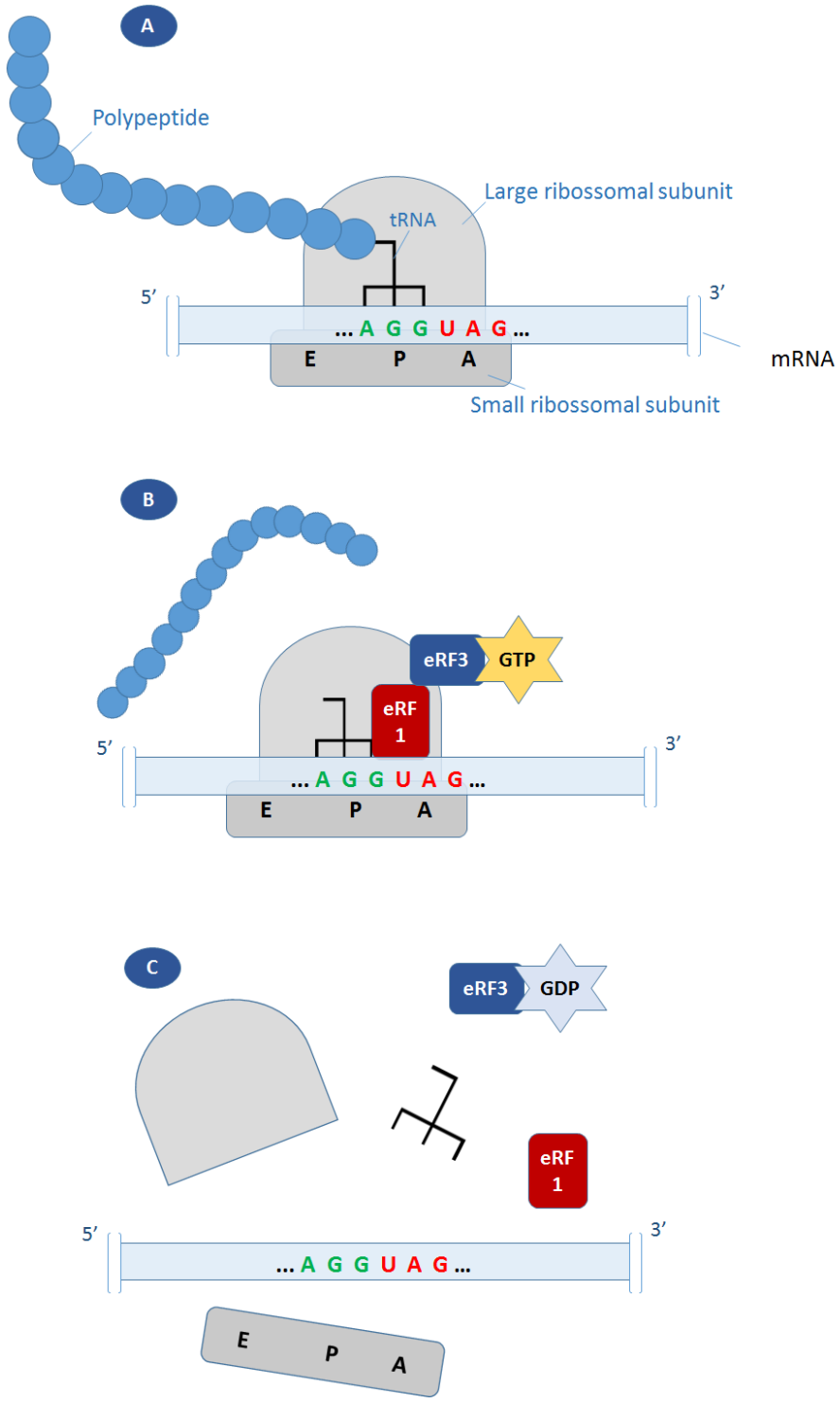


Figure 2: Eukaryotic translation termination. (A) There are no tRNA molecules that can bind with the stop codon (represented as UAG, in red) When a stop codon enters the ribosomal A-site, (B) eRF1/eRF3 complex binds to it, mimetizing a tRNA molecule. (C) eRF3 acts as a GTPase and hydrolyzes GTP into GDP and Pi. Thus, the translation machinery disassembles completely and the mRNA molecule is free to a new translation cycle. Adapted from Keeling *et al*., 2012.

1.3. Nonsense mediated mRNA decay

The mammalian nonsense mediated mRNA decay (NMD) is an mRNA surveillance mechanism. It has the function to degrade aberrant mRNA molecules. This gene expression surveillance mechanism is fast and highly conserved throughout the evolution of the species (Hall and Thein, 1994).

There are four classes of transcripts that trigger NMD:

- Alternative splicing (AS) resulting transcripts, wherein the AS event introduces a stop codon located at about 55 nucleotides upstream of the last exon-exon junction (Lareau *et al*., 2007; Saltzman *et al*., 2008);

- Normal splicing resulting transcripts, wherein the stop codon is located at about 55 nucleotides upstream of the last exon-exon junction (Maquat, 2004);

- Transcripts with long 3'-UTRs (Buhler *et al*., 2006; Singh *et al*., 2008);

- Transcripts containing short upstream ORFs (uORFs), because the uORF stop codon is recognized as premature (Oliveira and McCarthy, 1995).

Premature termination codons (also known as PTCs) are responsible for about a third of the human genetic disorders. The PTCs are originated, among other causes, due to nonsense and frameshift mutations in the DNA, which results in a premature termination of translation, producing truncated polypeptides. These polypeptides are shorter and, at most times, injurious for the cell. Some diseases may occur due to the lack of the full length proteins (Mühlemann *et al*., 2008).

The deposition of exon junction complexes (EJCs), 20 to 24 nucleotides upstream to the exon-exon junctions of the mRNA occurs during its processing (Le Hir *et al*., 2000). The Helicase eiF4AIII joins the heterodimer Y14/Magoh and allows the EJC attaching.

The EJCs function as a mark for the recognition of a stop codon as premature (Kozak, 1989; Hall and Thein, 1994; Lau *et al*., 2003; Le Hir *et al*., 2016).

During the pioneer round of translation, the ribosome removes the EJCs. If a PTC is detected more than 50 to 54 nucleotides upstream to the last exon-exon junction, the translation stops prematurely. If this happens, at least one EJC will remain associated to the mRNA molecule (Nagy *et al*., 1998; Silva and Romão, 2009; Schweingruber *et al*., 2013). As a result, the NMD is triggered and recruitment of exoribonucleases occurs. Exoribonucleases will degrade the mRNA. In the absence of PTCs, the ribosome reaches the natural termination codon, removing every single EJC stalled in the mRNA molecule, and a functional polypeptide is correctly synthetized. It may happen that if a PTC is located less than 50 to 54 nucleotides upstream to the last exon-exon junction, it will not be detected as premature. As a result, translation ends prematurely and a truncated peptide is synthetized, which may cause clinical conditions. (Nagy *et al*., 1998; Holbrook *et al*., 2004; Amrani *et al*., 2006; Silva and Romão, 2009; Schweingruber *et al*., 2013).

The UPF proteins (up-frameshift proteins) have an important role in the NMD. There are three types of UPF proteins: UPF1, UPF2 and UPF3. UPF2 and UPF3 proteins belong to the EJC. While the UPF3 binds to the mRNA during the splicing process, in the nucleus, the UPF2 protein binds to the UPF3 during the mRNA exportation through the nuclear pore (Lykke-Andersen *et al*., 2000; Serin *et al*., 2001; Kashima *et al*., 2006). In the cytoplasm, the interaction between the EJC and the translation termination complex promotes the interaction between the UPF2/UPF3 complex and the UPF1 protein (Serin *et al*., 2001; Kadlec *et al*., 2004; Kashima *et al*., 2006).

UPF1 proteins are mainly found hypophosphorylated in the cytoplasm. The interaction between UPF1 and the EJC has a direct influence in the translation termination. It has

been shown that CBP80, a protein found in the mRNA 5' cap, transiently interacts with the UPF1/SMG1 complex, during the initiation of pioneer round of translation. In yeast, this weak interaction is enough to promote the contact between the UPF1/SMG1 complex with eRF1 and eRF3, leading to the SURF complex formation. Therefore, the SURF complex is composed by UPF1, SMG1 (UPF1 kinase suppressor with morphogenetic effect on Genitalia 1), and release factors 1 and 3 (Lejeune *et al*., 2002; Kashima *et al*., 2006; Hwang *et al*., 2010; Choe *et al*., 2014). If a PTC is found in the mRNA molecule, the SURF complex binds it, and CBP80 elicits the connection between the UPF1/SMG1 complex and a PTC distal EJC, which has not been removed during the pioneer cycle of translation. Thus, UPF1 directly interacts with UPF2 and UPF3 bound to downstream EJC, and is activated by its kinase, SMG1, forming the decay-inducing complex (DECID). This chain of events subsequently represses the translation, and mRNA decay factors such as SMG5, SMG7 or PNRC2 (proline-rich nuclear receptor coactivator 2) and SMG6 are recruited. (Frolova *et al*., 1996; Wang *et al*., 2001; Kashima *et al*., 2006; Hwang *et al*., 2010; Peixeiro *et al*., 2011). These molecules will bind the phosphorylated UPF1, triggering the mRNA decay. SMG5, SMG6 and SMG7 contain an N-terminal domain consisting of 9 anti-parallel α-helices homologous to 14-3-3 proteins. 14-3-3 proteins are a group of proteins that bind molecules containing phosphorylated serines/threonines (Fukuhara *et al*., 2005; Obsil and Obsilova 2011; Jonas *et al*., 2013; Choe *et al*., 2014).

In metazoans, in order to occur NMD, UPF1 must be dephosphorylated. SMG5, SMG6 and SMG7 trigger UPF1 dephosphorylation by recruiting PP2A (phosphatase 2A) and NMD is triggered (figure 3). In humans, SMG6 promotes endonucleolytic cleavage of PTC-containing mRNA 5' to the EJC, in the PTC vicinity. It is the C-terminal PIN (Pil-T N-Terminus) domain present in SMG6 that is responsible for the mRNA cleavage.

(Page *et al.*, 1999; Denning *et al.*, 2001; Yamashita *et al.*, 2001; Anders *et al.*, 2003; Chiu *et al.*, 2003; Glavan *et al.*, 2006; Huntzinger *et al.*, 2008; Eberle *et al.*, 2009; Okada-Katsuhata *et al.*, 2011).

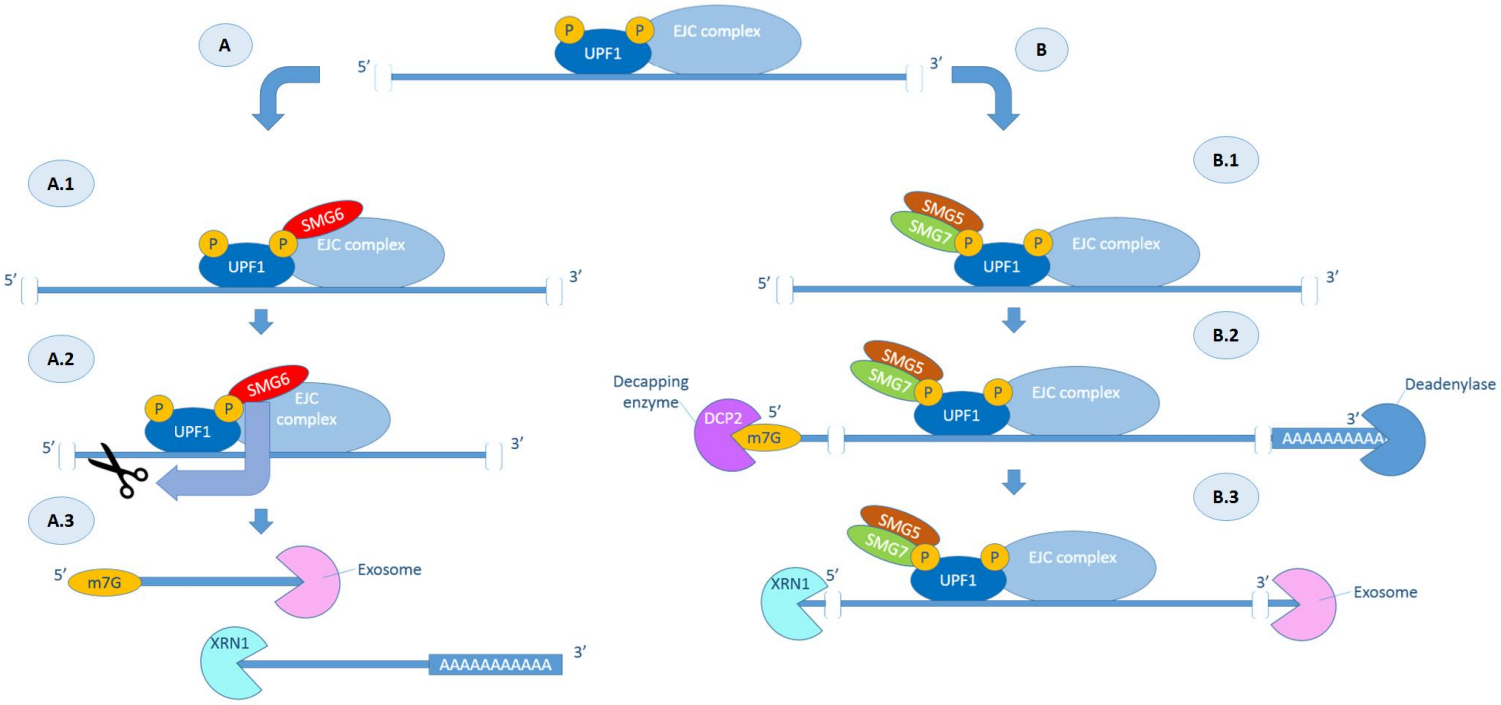


Figure 3: Model for degradation of NMD targets. UPF1-bound mRNA molecules may undergo through two different degradation pathways. (A) UPF1 may recruit SMG6. (A.1) SMG6 binds with phosphorylated UPF1 attached to a PTC-containing mRNA, (A.2) its C-terminal PIN domain endocleaves the mRNA molecule, 5' to the EJC. SMG6 has endocleavage activity. (A.3) Two decay intermediates result from the endocleavage. The 5' decay intermediate suffers 3'-to-5' digestion by the exosome and the 3' decay intermediate suffer 5'-to-3' digestion by the XRN1. (B) UPF1 may also recruit SMG5/SMG7 complex. SMG5/SMG7 complex has an important role in the degradation of PTC-containing mRNA molecules. SMG5 is an adaptor protein that forms a complex with SMG7 (B.1) N-terminus of SMG7 interacts with the phosphorylated UPF1 that binds PTC-containing mRNA molecules. (B.2) SMG7 brings UPF1 into P-bodies and recruits a decapping enzyme (DCP2), that digests the 5' cap, while deadenylases digest the mRNA Poly(A)tail. (B.3) Finally the mRNA molecule suffers 3'-to-5' digestion by the exosome and 5'-to-3' digestion by the XRN1. Adapted from Popp and Maquat, 2013.

SMG6 endocleavage generates 5' and 3' decay intermediates that suffer rapid decay by the exosome and the 5'-to-3' exoribonuclease 1 (XRN1) (figure 3.A). UPF1 also has helicase activity that is responsible for the disassembly of the RNP components bound

to the 3'-clevage product. (Gatfield and Izaurralde, 2004; Eberle *et al*., 2009; Popp and Maquat, 2013; Fiorini *et al*., 2015).

SMG7 uses its N-terminus to interact with the phosphorylated UPF1 protein and its C-terminus to elicit the mRNA decay. SMG7 usually brings UPF1 into P-bodies, where high concentrations of RNA decay factors can be found (Chang *et al*., 2007). SMG7 has also a major role in the recruitment of mRNA decay enzymes such as the DCP2, a decapping enzyme, and the exoribonuclease XRN1 (figure 3.B) (Unterholzner *et al*., 2004; Eulalio *et al*., 2007; Jonas *et al*., 2013; Loh, 2013).

If an mRNA molecule contains a PTC, the molecule may be committed to NMD, and its encoded protein may not be synthesized. In Mammalia, NMD is directly dependent on the splicing and translation machinery (Hall and Thein, 1994), and in general, it is not activated in mRNA synthetized from PTC-containing intronless genes. It has been shown that some PTC-containing mRNA molecules can evade the NMD process, thus being translated, which results in the formation of truncated proteins. The phenotype of an expressed truncated protein is usually more harmful to the cell than if the mRNA is degraded by the NMD process (Holbrook, *et al*., 2004). However, truncated proteins may have basal activity in the organism, as it has been shown in diseases like Duchenne muscular dystrophy (DMD), associated to some PTCs in the dystrophin gene (Dent *et al.*, 2005 and Malik *et al*., 2010), and cystic fibrosis, associated to some PTCs in the CFTR gene (Kerem *et al*., 2008 and Sermet-Gaudelus *et al*., 2010). Thus, in certain cases, despite the NMD protective role in the organism, its induction may be more deleterious than if it would not occur (Malik *et al*., 2010, Keeling and Bedwell, 2011 and Bartolomeo *et al*., 2013).

Eventually, PTCs at the β-globin gene may be responsible for some cases of β-thalassemia. However, it was shown that β-globin mRNA containing AUG-proximal

PTCs may not be taken for degradation (Romão *et al*., 2000). The same happens if a PTC is present in the third and last exon of the β-globin gene (Romão *et al*., 2000). For example, it has been shown by Salvatori and colleagues that human β-globin mRNA containing a PTC at codon 39 (β39 β-globin mRNA) (exon 2) is degraded by NMD, but β15 β-globin mRNA and β127 β-globin mRNA, both having PTCs, at the codon 15 (exon 1) and 127 (exon 3), respectively, evade NMD (Salvatori, Breveglieri *et al*. 2009; Salvatori, Cantale *et al*., 2009).

1.4. Suppression therapy

Molecules of tRNA that show complementarity to two of the three nucleotides of a stop codon are called near cognate aminoacyl tRNA molecules. The development of suppression therapies presupposes an increase in the competition of near cognate aminoacyl tRNA molecules with the eRF1/3 complex by the ribosomal A-site, therefore avoiding the premature termination of translation. When a stop codon enters the ribosomal A-site, release factors bind to it and the translation immediately ends. When a near cognate aminoacyl tRNA molecule binds a premature stop codon, it triggers a readthrough event. As such, the PTC is not recognized as a stop codon. Thus, the translation mechanism does not stop and another amino acid is incorporated into the growing polypeptide, which was not released from the translation machinery. (Keeling and Bedwell, 2011; Bartolomeo *et al*., 2013).

There are some ongoing studies that use antisense oligonucleotides, NMD inhibitor cofactors, suppressor tRNA molecules and aminoglycoside and non-aminoglycoside antibiotics, among others, aiming to establish a therapy for diseases due to PTCs (Malik *et al*., 2010, Goldmann *et al*., 2011; Tan *et al*., 2011; Ward *et al*., 2014).

It has been demonstrated that some aminoglycoside and some non-aminoglycoside molecules have the ability to suppress nonsense mutations as PTC readthrough compounds. These compounds increase the ability of near cognate aminoacyl tRNAs to compete with the translation release factors for the ribosomal A-site. Thus, mRNA translation does not end in the PTC and production of the full-length protein may occur (Mühlemann *et al.*, 2008; Keeling and Bedwell, 2011; Sanchez-Alcudia *et al.*, 2012; Keeling *et al.*, 2012, Keeling *et al.*, 2014).

Aminoglycosides are a class of antibiotics that contain amino sugar substructures. These antibiotics are used to inhibit bacteria translation (Mingeot-Leclercq et al, 1999). The ribosomal decoding center has a proofreading function, verifying the codon-anticodon interactions and ensuring the exclusive accommodation of cognate aminoacyl tRNA molecules in the peptidyl transferase center. The peptidyl transferase center is the structure where the peptide bonds are formed. It has been shown that aminoglycoside molecules can bind the ribosomal decoding center, present in the eukaryotic small ribosomal subunit, modifying its conformation, leading to a reduced ability to distinguish between tRNA substrates. This results in the misincorporation of near cognate aminoacyl tRNA in PTCs. Therefore, antibiotic molecules may enhance a readthrough effect during the mRNA translation (Lynch *et al*., 2001; Scheunemann *et al*., 2010; Lee *et al*., 2012; Gómez-Grau *et al*., 2015).

Readthrough effect can suppress the translation termination. However, not always the results are the same, depending on factors like the identity of the termination codon, the surrounding mRNA sequence context and the presence of stimulating compounds (Lee *et al*., 2012; Dabrowski *et al*., 2015). Other experiments should be performed in order to understand how readthrough capacity can be affected. Furthermore, when used in

patients, these compounds have revealed side effects like hearing loss and kidney damage (Malik *et al*., 2010; Prayle *et al*., 2010).

2. Aims

β-thalassemia is a genetic disease that may be caused due to nonsense mutations in the β-globin gene. One of the consequences of this, is the synthesis of a truncated peptide that may not have biological function. The aim of this study is to restore physiological levels of full-length β-globin protein and evaluate the response of β-thalassemia nonsense mutations to suppression therapy using increasing concentrations of aminoglycoside compounds, like kanamycin and gentamicin. It is also pretended to evaluate and differentiate between the readthrough efficiency of gentamicin and kanamycin. For that, HeLa cells were transiently transfected with plasmids containing a PTC-containing β-globin gene and their expression was studied recurring to Western blot and RT-qPCR.

3. Materials and Methods

3.1. Plasmid constructs

The wild-type β-globin, as well as the human β-globin variants β15 [CD 15 (TGG→TGA)] and β39 [CD 39 (CAG→TAG)], were cloned into the *ClaI*/*BspLU11I* sites of the pTRE2pur vector (BD Biosciences) carrying an ampicillin resistance gene, by overlap-extension PCR amplification of the 1806 bp *ClaI*/*BspLU11I* fragment, using primers with linkers for *ClaI* and *BspLU11I*, as described by Silva *et al.*, 2006. The sequence of the overlapping primers is shown in table 1.

Table 1: List of overlapping primers used in the β-globin gene cloning:

Primer	Sequence (5'→ 3')
#1	CCATCGATACATTTGCTTCTGACACAACTG
#2	TTACATGTAGGGATGGGCATAGGCATC

The plasmids were used to transform competent *Escherichia coli*. The transformant colonies were selected on ampicillin-containing agar and Luria-Bertani (LB) medium. The plasmid DNA was then isolated and purified by minipreps, using innuPREP Plasmid Mini Kit (Analytik Jena AG, Germany), following the protocol: "Isolation of high copy plasmid DNA from bacterial lysates", provided by the manufacturers.

In order to increase the yield of purified plasmid DNA, NZYMaxiPrep kit (Nzytech, Portugal) was also used, following the manufacturer's instructions.

The confirmation of the sequences was performed by automatic sequencing.

3.2. c-myc tag cloning

In this project, it was pretended to evaluate the effects of aminoglycosides, not only at the mRNA level, but also at the protein level. In previous assays, it has been problematic to detect the β-globin protein by Western blot, when using anti-HBB antibodies. In order to detect β-globin protein, a c-myc tag was added to the exon 3 of the β-globin gene of each type (βN, β15 and β39) cloned into the pTRE2pur vector.

Sense (#1) and antisense (#2) oligonucleotides, containing the c-myc tag sequence, were previously designed. The sequence of the synthetic oligonucleotides is shown in the table 2. The c-myc tag sequence is shown in green.

Table 2: List of oligonucleotides used in the c-myc tag cloning:

Oligonucleotide	Sequence (5'→ 3')
#1	TTGGCATGGAGCAGAAGCTGATCTCCGAGGAGGACCTGGCCCATCACT
#2	ATGGGCCAGGTCCTCCTCGGAGATCAGCTTCTGCTCCATGCCAAAGTG

A concentration of 22.5 pmol of each sense and antisense oligonucleotide was mixed with annealing buffer [final concentration: Tris/HCl (50 mM), pH 7.5; spermidine (1 mM); $MgCl_2$ (10 mM); DTT (5 mM)], in a total volume of 22.5 µL in microcentrifuge tubes (1 pmol corresponds nearly to 20 ng, for a single-stranded oligonucleotide containing 55 nucleotides). The annealing reaction was performed in a thermocycler (Biometra GmbH, Germany), with the conditions described in the table 3.

Table 3: Stages used in the annealing of two synthetic oligonucleotides to obtain a double-stranded DNA fragment:

Stage	Temperature	Time
#1	95ºC	5 minutes
#2	85ºC	10 minutes
#3	75ºC	10 minutes
#4	65ºC	10 minutes

The result was a double-stranded fragment at 1 pmol/µL (1 pmol/µL corresponds nearly to 40 ng/µL, for a double-stranded fragment containing 55 base pairs). A volume of 5 µL of the double-stranded fragment containing the c-myc tag sequence were submitted to electrophoresis in a 2% (w/v) SeaKem® LE Agarose (Lonza, Switzerland) gel, at 100 V. A volume of 10 µL of each single-stranded oligonucleotide were used as control.

Digestion of 2 µg of βN, β15 or β39 gene-containing plasmid was carried out using *BstXI* enzyme (NEB, USA), in a final volume of 50 µL, following the manufacturer's instructions. A volume of 10 µL of each digested product was loaded in a 0.8% (w/v) agarose gel and an electrophoresis was performed at 100 V. Non-digested plasmid DNA was used as control.

The double-stranded fragment carrying the c-myc tag was ligated to the *BstXI*-digested plasmid DNA using 1 U of T4 DNA ligase (Roche, USA), following the manufacturer's instructions, at room temperature, overnight.

The putative c-myc tag-containing plasmids were used to transform competent *Escherichia coli*. As before, the transformant colonies were selected on ampicillin-containing agar and Luria-Bertani (LB) medium. The plasmid DNA was isolated and purified as previously referred. The confirmation of the sequences was performed by automatic sequencing.

Initially there was no success in the ligation of the c-myc tag-containing double-stranded fragment into the *BstXI* site of the βN and β15 human β-globin gene-containing plasmids. Thus, once the c-myc tag was cloned into the *BstXI* site of the β39 plasmid, we used *NotI* and *BsrGI* restriction enzymes (NEB, USA) to remove the fragment containing the PTC at codon 39 (but not the c-myc tag), and replaced it by the corresponding fragment form βN and β15 genes. For that, 2 μg of β39-c-myc tag plasmid were digested using *NotI* and *BsrGI*, according to the manufacturer's instructions. Two μg of βN and β15 plasmids were digested with the same enzymes, in order to obtain a 965 bp *NotI*/*BsrGI* fragment containing no nonsense mutations and another 965 bp *NotI*/*BsrGI* fragment containing a nonsense mutation at codon 15 of the human β-globin gene, respectively. The fragments were separated by electrophoresis in a 0.8% (w/v) agarose gel, performed at 100 V. The isolation of the fragments was performed using the innuPrep Gel Extraction kit (Analytik Jena AG, Germany), following the manufacturer's instructions.

Both 965 bp isolated fragments containing the βN and β15 sequence were ligated into the *BsrGI*/*NotI* sites of the digested c-myc-tag-containing plasmid, using 1 U of T4 DNA ligase, following the manufacturer's instructions, at room temperature, overnight. The putative c-myc tag-containing plasmids were used to transform competent *Escherichia coli*, as mentioned before and the plasmid DNA isolation was performed as stated above.

3.3. Sequencing

The plasmids were sequenced with specific primers (table 4) in order to verify the β-globin gene sequence and in order to verify if the c-myc tag was cloned in frame.

Table 4: List of primers used for Automatic sequencing:

Primer	Sequence (5'→ 3')
#1	ACATTTGCTTCTGACACAAC
#2	AACGGAATTGGGTC
#3	CCTAATCTCTTTCTT
#4	AGCTCGCTTTCTTGCTGTCC
#5	CCTTGATACCAACCTGCCCA

For each sample a PCR mix containing 300 ng of plasmid, 1 µL of primer (2 µM), 1 µL of BigDye (Thermo Fisher Scientific, USA) and water added to a final volume of 10 µL, was prepared, and incubated in a thermocycler (Biometra GmbH, Germany), with the following cycle conditions: 96°C for 45 seconds, followed by 25 cycles of 96°C for

20 seconds, 55ºC for 5 seconds and 60ºC for 4 minutes. The amplified samples were sequenced by automatic sequencing.

3.4. Cell culture

HeLa cells were carefully cultured in DMEM (Dulbecco's modified Eagle's medium 1x + GlutaMAX-I; Gibco by Life Technologies, USA) supplemented with 10% (v/v) FBS (Fetal bovine serum; Gibco by Life Technologies, USA), in a humidified atmosphere of 5% CO_2 incubator at 37ºC. Cells in the mid-log growth phase were used in the following research.

3.5. Transient transfection

Mid-log grown HeLa cells were transfected with 3 µg of plasmid containing its respective β-globin gene variant, in 35 mm tissue culture dishes.

Opti-MEM medium (Gibco by Life Technologies, USA) was used as transfection medium; Lipofectamine 2000 Transfection Reagent (Invitrogen by Life Technologies, USA) was used as transfection reagent, to transfect HeLa cells, following the manufacturer's instructions. The plates were incubated at 37ºC during 24 hours.

3.6. Drug treatment

In order to prepare gentamicin and kanamycin stock solutions at 50 mg/mL, 75.2 mg of gentamicin (Nzytech, Portugal) were diluted in 1,504 mL of water and 98.2 mg of kanamycin (monosulphate, Nzytech, Portugal) were diluted in 1.960 mL of water, respectively.

Twenty four hours after transfection, HeLa cells were treated with either gentamicin or kanamycin at different concentrations (0 µg/mL, 10 µg/mL, 100 µg/mL and 1000 µg/mL). The different drug solutions were prepared by diluting the drug in DMEM supplemented with 10% (v/v) FBS. Each culture dish had its medium removed and replaced with the new prepared medium supplemented with its respective drug concentration.

3.7. Cell lysis

After 24 hours of drug treatment, cells were lysed with NP-40 [Tris-HCl (50 mM, pH 7.5); $MgCl_2$ (2 mM); NaCl (100 mM); Glycerol (8.64%, v/v); NP-40 (1% v/v), Roche, USA]. Cells were washed with 1x PBS (Phosphate-buffered saline). After PBS removal, the cells were incubated with NP-40 buffer. Then, the samples were harvested and collected in microcentrifuge tubes and centrifuged at 13200 rpm for 2 minutes. The supernatant was transfered in new microcentrifuge tubes and the pellet was discarded. Samples were stored at -80ºC.

3.8. RNA isolation

Total RNA extraction and purification was performed using the NucleoSpin RNA II kit (Macherey-Nagel, Germany), following the manufacturer's instructions. The RNA samples were stored at -80ºC.

3.9. Quantitative reverse transcription PCR (RT-qPCR)

The cDNA synthesis from 1 µg of total RNA was performed recurring to the NZY Reverse Transcriptase kit (Nzytech, Portugal). For each sample, 1 µg of total RNA, 1 µL of random hexamer primers (250 ng/µL), 1 µL of dNTP (10 mM) and water added to 16 µL, were incubated for 5 minutes at 65ºC. The samples were placed on ice and 2 µL of 10x RT reaction buffer (Nzytech, Portugal), 0.1 µL of NZY Ribonuclease inhibitor (Nzytech, Portugal), 0.5 µL of NZY Reverse Transcriptase (Nzytech, Portugal) and 1.4 µL of water were added and mixed. Each sample was incubated in a thermocycler. The cycling conditions are mentioned in the table 5.

Table 5: Cycling conditions used in cDNA synthesis:

Stage	Temperature	Time
Preincubation	25ºC	10 minutes
Incubation	50ºC	50 minutes
Reaction inactivation	85ºC	5 minutes

For quantitative PCR, cDNA was diluted 1:10 with water in a final volume of 40 µL. The SybrGreen Master Mix kit (Applied Biosystems by Life Technologies, USA) was used. The reaction conditions consisted of 5 µL of cDNA diluted as mentioned above, 7 µL of Sybr Green Master Mix and 1 µM of each primer (table 6) in a final volume of 15 µL. Quantitative PCR was carried out in the 7500 Real-Time PCR System (Applied Biosystems by Life Technologies, USA). The cycle conditions for quantitative PCR were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 62°C for 30 seconds. The Puromycin resistance ($Puro^R$) mRNA was used as an internal control.

Table 6: List of primers used in the quantitative PCR:

Gene	Primer Forward (5'→ 3')	Primer Reverse (5'→ 3')
β-globin	GTGGATCCTGAGAACTTCAGGC	CAGCACACAGACCAGCACGT
Puromycin Resistance	GGGTCACCGAGCTGCAAGAA	CACACCTTGCCGATGTCGAG

Quantification was carried out by the relative standard curve method ($\Delta\Delta C_t$, Applied Biosystems by Life Technologies, USA).

3.10. Western blot

A Western blot assay was performed in order to detect the β-globin protein. The α-tubulin protein was used to control the amount of loaded protein in each case (loading control).

The proteins of the lysed HeLa cells samples were homogenized in 4 µL of sample buffer 5x [Tris-HCl (200 mM, pH 6.8); Glycerol (25%, v/v); SDS (25%, w/v); DTT 525mM; bromophenol blue (0.25%, w/v)], and denatured at 95ºC, 10 minutes, in a final volume of 20 µL.

The separation of the proteins was performed by SDS-PAGE. The current intensity was fixed at 20 mA. Table 7 shows the constitution of the polyacrylamide gel.

Table 7: Polyacrylamide gel constitution:

Solutions	Running gel (14%)	Stacking gel (4%)
H_2O (mL)	1.95	1.5
Lower Buffer (mL)	1.25	-
Upper Buffer (mL)	-	0.25
Acrylamide 40% (w/v) (mL)	1.75	0.2
SDS 10% (w/v) (mL)	0.05	0.02
APS (µL)	50	50
TEMED (µL)	5	5

After the polyacrylamide gel electrophoresis, the gel was kept in contact with a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). The separated proteins were blotted onto the membrane at a fixed electric potential difference of 100 V, for 1 hour. Blot buffer (1x) [SDS (1.29 M); Tris (48 mM); glycine (38.7 mM); methanol (20%, v/v)] was used in order to perform the blotting step.

A staining step was performed, using a Coomassie Blue solution [glacial acetic acid (10%, v/v); methanol (45%, v/v); Brilliant Blue G (Sigma-Aldrich, USA) (2.93mM)]. The destaining of the PVDF membrane was carried out by using a destaining solution [Methanol (45%, v/v); Acetic Acid (10%, v/v)] and three washing steps were performed by using TBS-Tween20 solution (TBS (1x); Tween 20 (0.1%, v/v); Sigma-Aldrich, USA).

A blocking solution containing TBS-Tween 20 and milk powder (5%, w/v, Molico, Nestlé, Switzerland), was used to block nonspecific molecules in the membrane, for one hour.

Primary antibodies were diluted in the same blocking solution mentioned above. The membranes were incubated with a 1:10000 dilution of the anti-α-tubulin antibody (Roche, Switzerland) and a 1:400 dilution of the anti-HBB antibody (Sigma-Aldrich, USA) overnight.

A triple washing using TBS-Tween20 solution was carried out after the overnight primary antibody probing. A 1:4000 dilution of the secondary antibody anti-mouse IgG HRP (Bio-Rad, USA) was prepared for both cases. The membranes were then incubated with the secondary antibody for 1 hour and the enhanced chemiluminescence reaction (ECL) was carried out after another triple washing step, using TBS-Tween20 solution. The exposure times that were used were 5 minutes, 2 minutes, 1 minute and 1 second.

In order to detect c-myc-tagged β-globin, an overnight incubation with a 1:100 dilution of the anti-c-myc-tag antibody (Sigma-Aldrich, USA) was performed. As secondary antibody, a dilution of 1:3000 of anti-rabbit IgG HRP (Bio-Rad, USA) was prepared. The membrane was incubated with the secondary antibody for 1 hour.

3.11. Statistical analysis

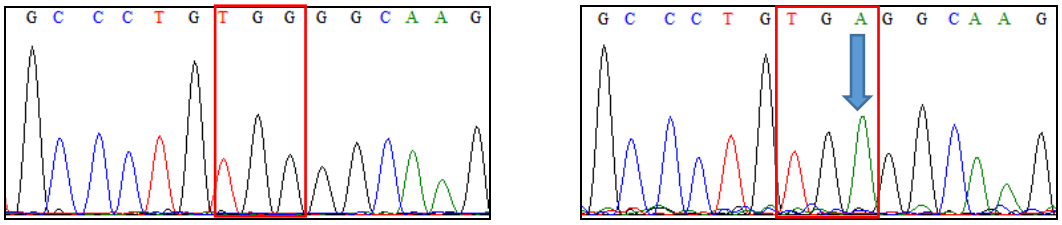
Microsoft Office Excel 2013 (Microsoft, USA) and Prism GraphPad 6.01 (GraphPad Software, Inc., USA) softwares were used for statistical analysis.

In order to analyze RT-qPCR data, two different normalizations were carried out: In the first case, the level of each mutant β-globin mRNA was normalized to the wild-type β-globin mRNA level, at the drug concentration of 0 μg/mL. In the other case, the level of each variant β-globin mRNA, at one specific concentration of drug, was normalized to the wild-type β-globin mRNA level, at the same concentration of the drug. In order to detect statistical significance of data, unpaired Student's t test was performed. Differences were considered as significant if $p<0.05$.

4. Results and Discussion

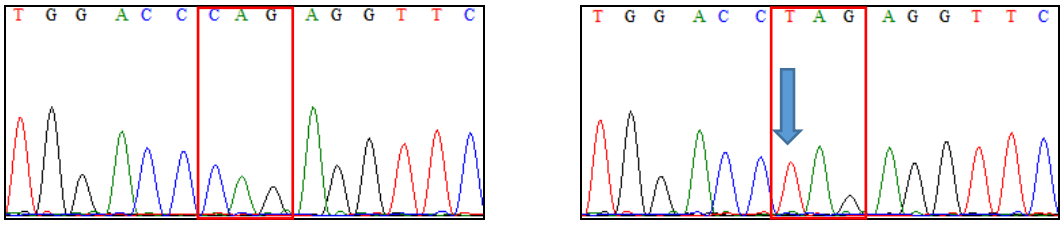
4.1. Constructs

In order to verify the sequence of either βN, β15 or β39 human β-globin gene previously cloned in the pTRE2pur plasmids (as described in Silva *et al*., 2006), a sequencing reaction was carried out. Recurring to the software BioEdit Sequence Alignment Editor (Ibis Biosciences, USA), the resulting sequences were analyzed. The presence of unwanted mutations was evaluated for each gene. The presence of the nonsense mutations at codons 15 and 39, in the β15 gene and the β39 gene, respectively, were also analyzed. The flanking plasmid sequences were also analyzed. It was concluded that the normal β-globin gene (βN) did not carry any mutation, β15 gene carrying the nonsense mutation at the codon 15 [CD 15 (TGG→TGA)], did not have other mutations (figure 4), and β39 gene carrying the nonsense mutation at the codon 39 [CD 39 (CAG→TAG)], was also intact (figure 5).



A. βN B. β15

Figure 4: Partial electropherogram of amplified βN and β15 human β-globin gene (forward). The red square indicates the β-globin gene codon 15. The vertical arrow points to the changed nucleotide. (A) Amplified βN β-globin gene sequence. The codon 15 sequence is 5'-TGG-3'. (B) Amplified β15 β-globin gene sequence. The codon 15 is a PTC and its sequence is 5'-TGA-3'.



A. βN B. β39

Figure 5: Partial electropherogram of amplified βN and β39 human β-globin gene (forward). The red square indicates the β-globin gene codon 39. The vertical arrow points to the changed nucleotide. (A) Amplified βN β-globin gene sequence. The codon 39 sequence is 5'-CAG-3'. (B) Amplified β39 β-globin gene sequence. The codon 39 is a PTC and its sequence is 5'-TAG-3'.

To clone the c-myc tag in the exon 3 of the of the βN, β15 and β39 genes, sense and antisense oligonucleotides containing the c-myc tag sequence were annealed. The annealing success can be observed in the figure 6.

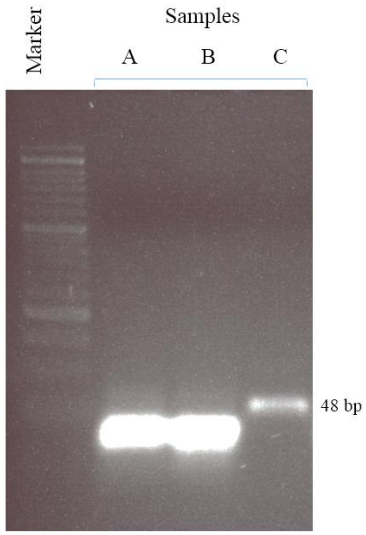


Figure 6: Agarose gel (2%, w/v) showing the success of the annealing of sense and antisense oligonucleotides containing the c-myc tag sequence. (A) Sense oligonucleotide (control). (B) Antisense oligonucleotide (control). (C) Annealed double-stranded DNA fragment containing the c-myc tag sequence. NZYDNA Ladder VI (Nzytech, Portugal) was used as molecular weight marker.

In order to insert the c-myc tag, *BstXI* restriction enzyme was used to digest the β-globin containing plasmids. The results of the digestion are shown in the figure 7.

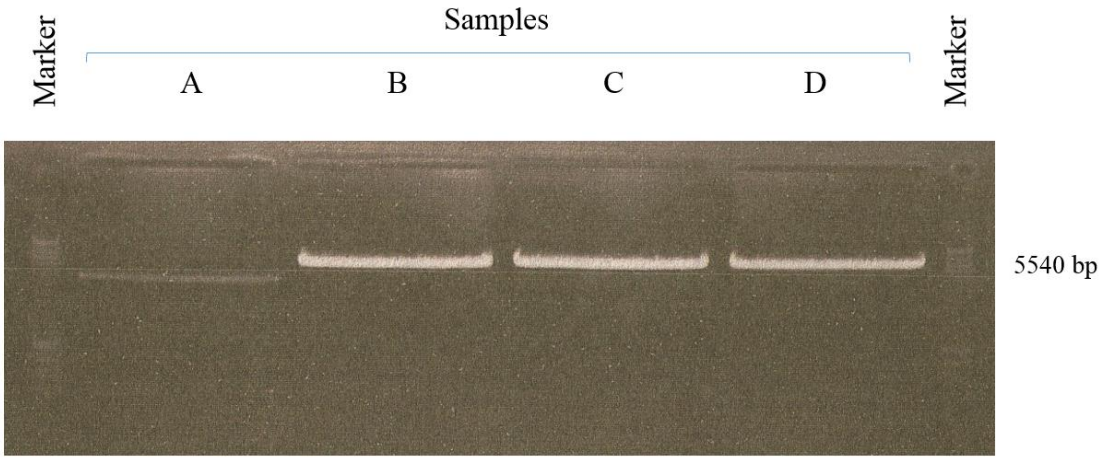


Figure 7: Agarose gel (0.8%, w/v) showing the fragments obtained by the digestion of (B) βN, (C) β15 and (D) β39 β-globin gene-containing plasmid with *BstXI*. (A) Uncut plasmid DNA (control). NZYDNA Ladder III (Nzytech, Portugal) was used as molecular weight marker.

Once we were only successful in the ligation of the c-myc tag-containing double-stranded fragment into the *BstXI* site of the β39 plasmids, but not into the *BstXI* site of the βN and β15 plasmids, we used *NotI* and *BsrGI* restriction enzymes to digest βN, β15 and β39 plasmids. The results of the digestion are shown in the figure 8.

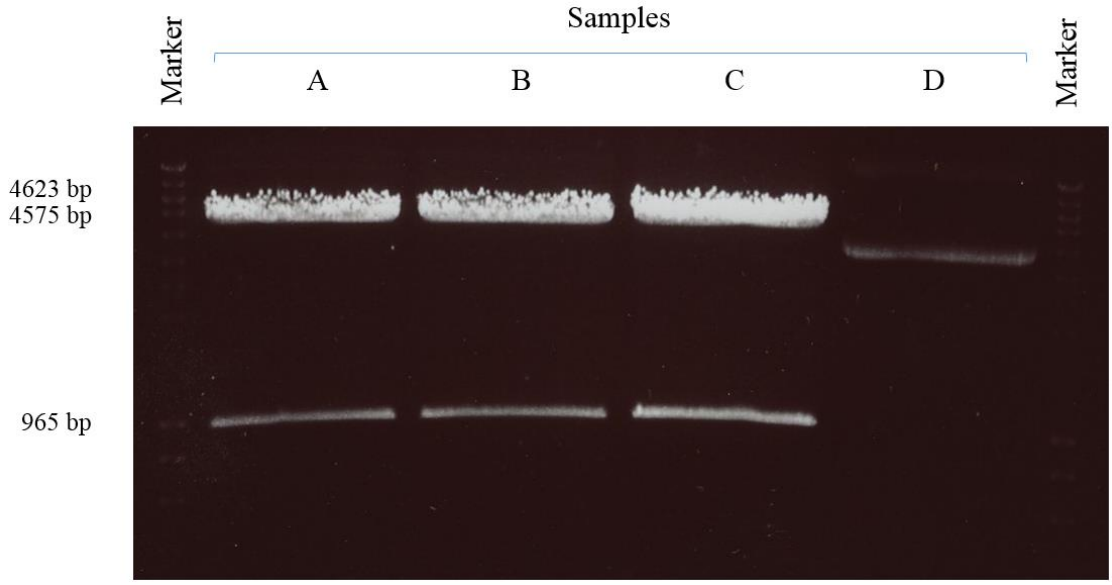


Figure 8: Agarose gel (0.8%, w/v) showing the fragments of βN and β15 β-globin gene-containing plasmids and c-myc tagged-β39 β-globin gene-containing plasmids digested with *NotI* and *BsrGI*.

NZYDNA Ladder III (Nzytech, Portugal) was used as molecular weight marker. (A) Digestion of βN β-globin gene-containing plasmid resulted in two fragments of 4575 bp and 965 bp. (B) Digestion of β15 β-globin gene-containing plasmid resulted in two fragments of 4575 bp and 965 bp. (C) Digestion of c-myc tagged-β39 β-globin gene-containing plasmid resulted in two fragments of 4623 bp and 965 bp. (D) Uncut plasmid DNA (control).

NotI and *BsrGI* were used to remove a fragment containing the PTC at codon 39 (but not the c-myc tag), in the β39 plasmid. The 965 bp *NotI*/*BsrGI* fragments containing either the βN or β15 sequence were ligated using DNA ligases to the 4623 bp resulting fragment containing the c-myc tag sequence. The whole β-globin gene sequence was sequenced with specific primers in order to verify if the c-myc tag was cloned in frame. Sequence analysis confirmed that the c-myc tag was correctly added to the codon 118 of the βN, β15 and β39 β-globin gene. Its sequence is shown in figure 9.

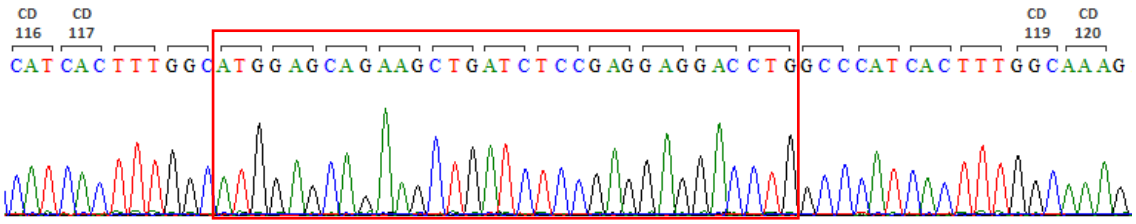


Figure 9: Partial electropherogram of amplified β-globin gene (forward) showing that the c-myc tag is in frame with the β-globin gene. The red square indicates the c-myc tag sequence.

4.2. Analysis of the expression levels of the wild-type β-globin mRNA, and its variants (β15 and β39) by RT-qPCR

It was demonstrated that the level of the β-globin mRNA with a nonsense mutation at codon 15 is very similar to the level of expression of the wild-type β-globin, indicating that it resists to NMD (Silva *et al*., 2008). Moreover, it was demonstrated that the level of the β-globin mRNA with a nonsense mutation at codon 39 is lower than the expression of the wild-type mRNA, showing that it is committed to NMD (Romão *et al*., 2000). In order to confirm these results, HeLa cells were transiently transfected with βN, β15 or β39 plasmids. Twenty-four hours after transfection, cells were harvested and total RNA was purified. Thus, RT-qPCR was carried out to quantify the relative β-globin mRNA levels. Each mRNA level was normalized to the β-globin wild-type mRNA, as shown in figure 10.

Results show that the level of β15 mRNA corresponds to 83% of the expression of the wild-type mRNA, and the β39 mRNA corresponds to 21% of the βN expression level. These results are in accordance with those previously described in Romão *et al*., 2000, Salvatori, Breveglieri *et al*. 2009 and Salvatori, Cantale *et al*., 2009.

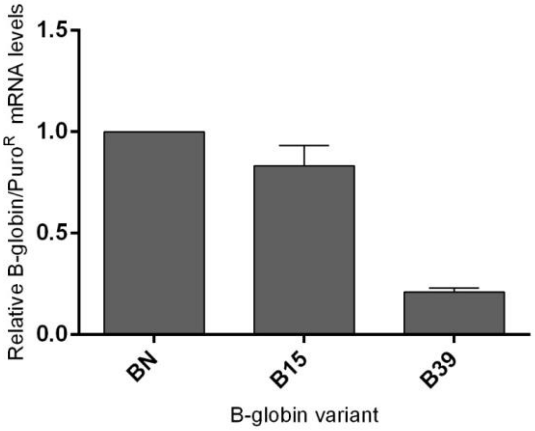


Figure 10: Expression levels of the wild-type (BN) and variants (B15 and B39) β-globin mRNA expressed in HeLa cells and quantified by RT-qPCR. β15 and β39 mRNA levels were normalized to those of the wild-type β-globin mRNA.

4.3. β-globin protein analysis by Western blot

The suppression therapy aims to increase the amount of full-length protein expressed from genes carrying PTCs. In order to monitor de the translation of the transfected mRNAs into the respective proteins, HeLa cells were transiently transfected with βN, β15 and β39 plasmids. Twenty-four hours after transfection, the cells were lysed with NP-40. The lysates were used in a Western blot assay, using antibodies to detect the β-globin protein.

The β-globin resulting signal was very faint and, in some assays, even inexistent. The lack of signal may have been due to some experimental steps like transfection, HeLa cell lysis, Western blotting or even the expression of the protein itself. Thus, some alterations to the initial protocol were carried out.

Initially an amount of 500 ng of plasmid was used to transiently transfect mid-log grown HeLa cells. It was hypothesized that the amount of transfected plasmid was low. So, instead of 500 ng, 3 µg of plasmid were transfected in mid-log grown HeLa cells. An increase of the quantity of the lysed samples was also carried out. Duplicate 35 mm tissue culture dishes were prepared. Thus, the same plasmid sample was transfected in every two plates. 100 µL of NP-40 were used in order to lyse the transfected cells. After obtaining the lysed samples, each different sample was transported to its duplicate plate and used to lyse its cells, which increased the lysed cells quantity.

The purpose of the c-myc-tagged constructs was to detect the β-globin protein using an anti-c-myc-tag antibody, instead of using anti-HBB antibodies. The length of c-myc tagged β-globin is 17 kDa, instead of 16 kDa. However, after some attempts, it was concluded that not even the c-myc-tagged β-globin could be detected with anti-c-myc antibodies, as shown in the figure 11.

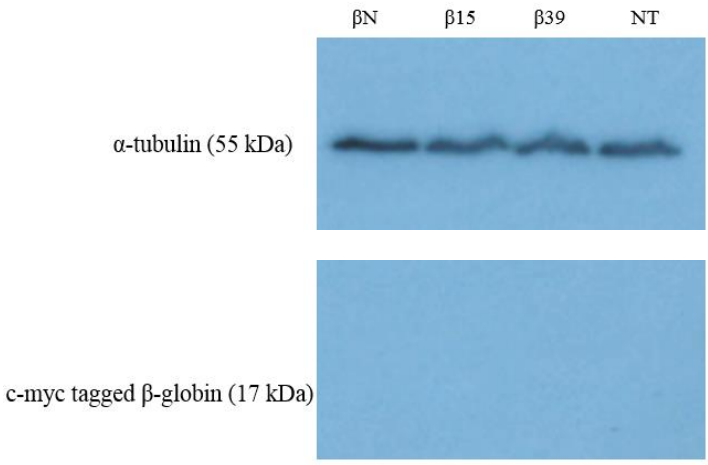


Figure 11: Analysis of the c-myc tagged β-globin (17 kDa) by Western blot. The α-tubulin (55 kDa) was used as control. NT corresponds to the proteins from non-transfected cells. Anti-c-myc antibody was used as primary antibody in order to detect c-myc tagged β-globin. Anti-α-tubulin antibody was used as primary antibody in order to detect α-tubulin.

An experiment to detect a different c-myc-tagged protein permitted to conclude that the primary antibody was not the cause of the lack of c-myc-tagged β-globin signal, because that protein signal was visible. However, c-myc-tagged β-globin signal was not detected and anti-HBB antibody was used again.

It was hypothesized that because the β-globin proteins are too small (16 kDa), their low signal may have had to do with the blotting step. In order to increase the blotting step efficiency, Towbin buffer [Tris (0.025 M); glycine (0.192 M), methanol (20%)] was used, instead of blot bluffer 1. The blotting time was 30 minutes instead of 1 hour. However, no alteration was observed and there was no β-globin protein signal. Assays with increased exposure time (up to 10 minutes) were carried out. The β-globin signal remained weak or non-existent.

Every Western blot solution had their formula verified. It was noticed that the SDS concentration that was being used in the blot buffer 25x was wrong, and its formula was corrected, as shown in Table 8.

Table 8: Rectification of the blot buffer 25x formula:

Solutions	Wrong formula	Amended formula
SDS	4.5 mM	32.25 mM
Tris	1.2 M	1.2 M
Glycine	0.97 M	0.97 M

It was also hypothesized that the β-globin low signal was due to the viscosity of the lysates, due to DNA contamination. So, in order to reduce the lysed cells samples viscosity, instead of using NP-40, the lysis step was performed with Sample buffer and Benzonase, a nuclease that hydrolyzes DNA and RNA molecules, which reduces the viscosity of the lysed samples. A Sample buffer/Benzonase lysis solution [Sample buffer (2x); $MgCl_2$ (0.01 M); Benzonase (0.5 U/µL)] was prepared. The Benzonase optimal temperature is 37ºC, so, the lysis was performed at room temperature and then, the samples were incubated at 37ºC, for 10 minutes. However, the results maintained the same and the protein signal remained too weak.

To test whether the amount of primary antibody was not enough, its concentration was also increased from a dilution of 1:400 to 1:250. Both β-globin and c-myc tagged β-globin were detected with anti-HBB antibody in the same experiment, as shown in the figure 12, however, despite all efforts, the β-globin protein still did not appear as pretended, and its signal remained too weak.

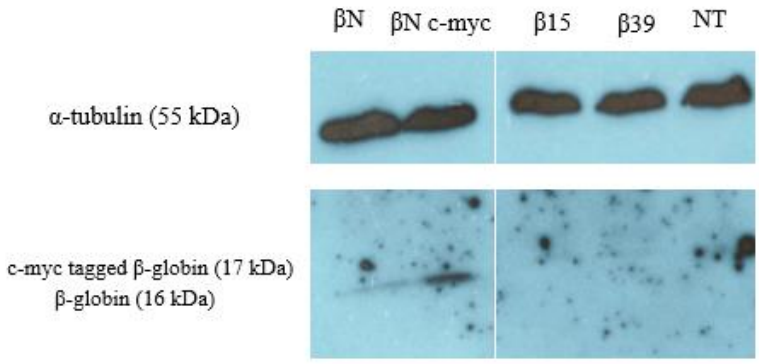


Figure 12: Analysis of the β-globin (16 kDa) and c-myc tagged β-globin (17 kDa) by Western blot. The α-tubulin (55 kDa) was used as control. NT corresponds to the proteins from non-transfected cells. Anti-HBB antibody was used as primary antibody in order to detect β-globin. Anti-α-tubulin antibody was used as primary antibody in order to detect α-tubulin.

In order to get expression of the β-globin gene, cells that usually express β-globin, like murine erythroid cells should be used. A tag should be inserted in the β-globin gene in order to distinguish between the native and the exogenous β-globin protein.

4.4. Analysis of the expression levels of the wild-type β-globin mRNA, and its variants (β15 and β39), when exposed to aminoglycosides

One of the aims of this project, was to evaluate the effect of increasing concentrations of aminoglycosides in transiently transfected HeLa cells on the suppression of nonsense mutations. For that, βN, β15 or β39 human β-globin gene-containing plasmids were transfected into HeLa cells. Twenty-four hours after transfection the cells were treated with different concentrations (0, 10, 100 or 1000 μg/mL) of either gentamicin or kanamycin for 24 hours. The cells were harvested and RNA purification was performed. Thus, RT-qPCR was carried out to quantify the relative β-globin mRNA levels.

In order to obtain more information from data obtained by RT-qPCR, for each drug, two distinct normalizations (which I will call Normalization A and B, respectively, from

now on) were carried out. In the Normalization A, each β-globin mRNA level was normalized to the βN mRNA expressed in HeLa cells without drug treatment (βN.0). In the Normalization B, β15 and β39 mRNA expressed in HeLa cells exposed to a certain drug concentration, were normalized to the wild-type mRNA expressed in HeLa cells in the corresponding conditions.

The major difference between the two performed normalizations is that in the Normalization A, every transcript is compared to a control that was not treated, whereas in the Normalization B, every PTC-containing transcript is compared to the same control treated with the same concentration of drug. In this way, it may be possible to understand if the drug is harmful, neutral or beneficial to the β-globin mRNA synthesis.

4.4.1. Gentamicin – Normalization A

RT-qPCR results show that the increase of gentamicin concentration may slightly enhance the βN gene expression, as βN mRNA levels in HeLa cells exposed to 10 μg/mL, 100 μg/mL and 1000 μg/mL of gentamicin, correspond respectively to 138%, 110% and 111% of the wild-type mRNA expressed in HeLa cells without treatment (βN.0) (figure 13). This suggests that gentamicin does not have harsh effects in the expression of the wild type β-globin gene.

Gentamicin seems to have a very slight effect in the β15 mRNA level, at concentrations of 10 and 100 μg/mL, as the mRNA level corresponds to 115% and 119% of the wild-type mRNA (βN.0). In this assay, the expression level of the β15 mRNA expressed in HeLa cells without gentamicin treatment was 82% of the wild-type mRNA expression level (βN.0). HeLa cells transfected with β15 gene grown in DMEM with 1000 μg/mL of gentamicin do not seem to be affected, once its mRNA expression level corresponds

to 81% of the wild-type mRNA (βN.0) (figure 13). Gentamicin does not also seem to negatively affect the β15 gene expression.

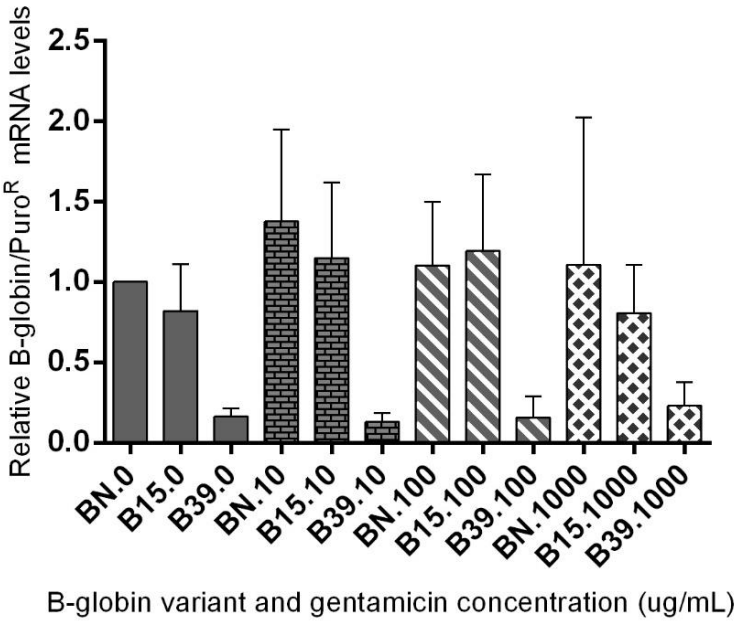


Figure 13: Analysis by RT-qPCR of wild-type (BN) and variants (B15 and B39) β-globin mRNA in HeLa cells treated with different concentrations of gentamicin (0, 10, 100 and µg/mL). The expression level of each β-globin mRNA was normalized to the wild-type β-globin mRNA level expressed in HeLa cells without gentamicin treatment.

The expression level of β39 transcripts, expressed in HeLa cells treated with 0, 10, 100 and 1000 µg/mL of gentamicin were, respectively, 16%, 13%, 16% and 23% of the wild-type transcripts expressed in HeLa cells without treatment (βN.0) (figure 13). The main goal of this study is to check if aminoglycoside drugs have any effect on suppressing NMD of nonsense codon-containing transcripts. As mRNA molecules are directly linked to the protein synthesis, their expression study is fundamental. However, no significant increase was noticed (figure 13). Although, there was a slight increase in the β39 transcripts expressed in HeLa cells treated with 1000 µg/mL of gentamicin, it was not significant. Besides that, the standard deviation is too high. Further experiences

should be carried out in order to reduce the deviation standard and understand if there is any positive effect in the β39 mRNA levels.

4.4.2. Gentamicin – Normalization B

Without gentamicin treatment, the β15 and β39 transcripts are respectively expressed at 82% and 16% of the βN mRNA levels (βN.0) (figure 14).

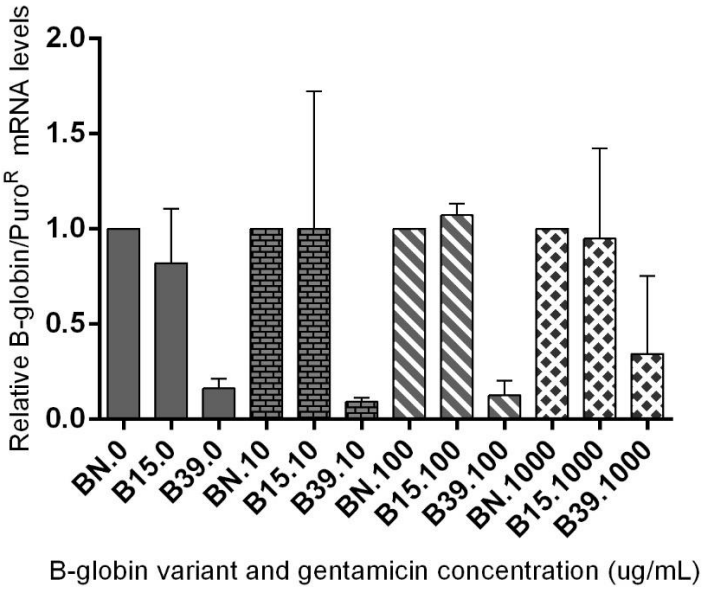


Figure 14: Analysis by RT-qPCR of wild-type (BN) and variants (B15 and B39) β-globin mRNA in HeLa cells treated with different concentrations of gentamicin (0, 10, 100 and μg/mL). Each β15 or β39 β-globin mRNA expression level, at a specific gentamicin concentration, was normalized to the wild-type β-globin mRNA level, at the same conditions.

When HeLa cells were treated with gentamicin at 10 μg/mL, β15 and β39 mRNAs are respectively expressed at 100%, (although with a very huge standard deviation). and 9% of the wild-type transcripts (βN.10), at the same conditions, which suggests a little decrease in the β39 β-globin mRNA (figure 14). This scenery remains almost the same at the concentration of 100 μg/mL of gentamicin, as the expression level of β15 and β39 transcripts are, respectively 107%, although with a much smaller standard deviation than the last one, and 12% of the wild-type transcripts (βN.100) (figure 14).

Something interesting can be noticed at the concentration of 1000 µg/mL of gentamicin; although the expression level of β15 transcripts is very similar to the predecessor gentamicin concentrations (95% of the wild-type transcripts), the level of expression of β39 transcripts is 34% of the βN transcripts (βN.1000), which is rather higher than the level of previous β39 transcripts. Unfortunately, the standard deviation is too high, and this value is not significant. With this information, further experiments using gentamicin at 1000 µg/mL would be interesting to perform, in order to check whether β39 mRNA levels increase.

4.4.3. Kanamycin – Normalization A

RT-qPCR results show that the increase of kanamycin concentration may maintain or slightly enhance the βN gene expression, as βN mRNA levels in HeLa cells exposed to 10 µg/mL, 100 µg/mL and 1000 µg/mL of kanamycin, correspond to 100%, 117% and 147% of the wild-type mRNA expressed in HeLa cells without kanamycin treatment (βN.0), respectively (figure 15). Kanamycin is an aminoglycoside that does not seem to be interfering negatively with the expression of the human β-globin gene.

At the concentrations of 0, 10, 100 and 1000 µg/mL of kanamycin the expression level of β15 transcripts is 118%, 88%, 83% and 168% of the βN transcripts (βN.0) respectively (figure 15). β15.1000 transcripts appear to be overexpressed (figure 15).

Kanamycin does not seem to play any role on β39 transcripts, once their expression level, when compared to the βN transcripts (βN.0), is 22%, 24%, 18%, 22% at the concentrations of 0, 10, 100 and 1000 βg/mL, respectively (figure 15). In order to evaluate if kanamycin has readthrough effect on β39 transcripts, different kanamycin concentrations could be used.

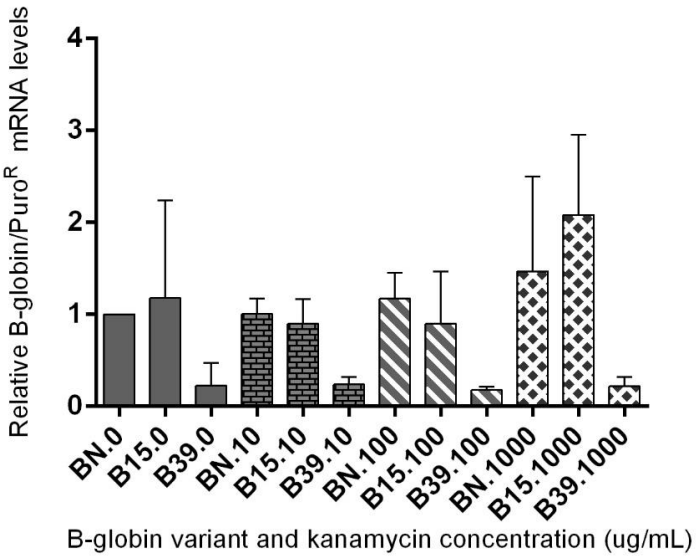


Figure 15: Analysis by RT-qPCR of wild-type (BN) and variants (B15 and B39) β-globin mRNA in HeLa cells treated with different concentrations of kanamycin (0, 10, 100 and µg/mL). The expression level of each β-globin mRNA was normalized to the wild-type β-globin mRNA level expressed in HeLa cells without kanamycin treatment.

4.4.4. Kanamycin – Normalization B

The expression levels of β15 and β39 transcripts from HeLa cells without kanamycin treatment is, as it has been mentioned before, 118% and 22% of the βN transcripts level (βN.0), respectively (figure 16).

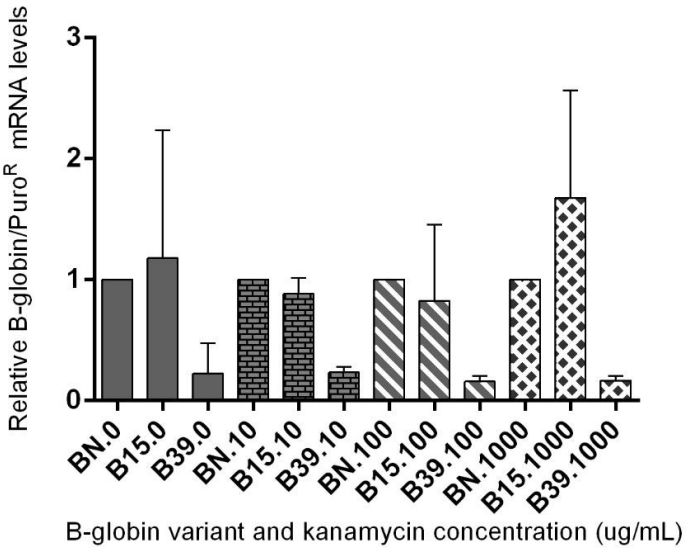


Figure 16: Analysis by RT-qPCR of wild-type (BN) and variants (B15 and B39) β-globin mRNA in HeLa cells treated with different concentrations of kanamycin (0, 10, 100 and μg/mL). Each β15 or β39 β-globin mRNA expression level, at a specific kanamycin concentration, was normalized to the wild-type β-globin mRNA level, at the same conditions.

At the kanamycin concentration of 10 μg/mL, the expression levels of β15 and β39 transcripts are respectively 88% and 23% of the expression level of the wild-type transcripts (βN.10) at the same conditions (figure 16). This data shows that at this kanamycin concentration, there are no major changes in both PTC-containing transcript levels. The same happens when cells are treated with a kanamycin concentration of 100 μg/mL of kanamycin. The transcripts show an expression level of 83% and 16% of the wild-type mRNA (βN.100) levels, although the standard deviation is much higher in the β15.100 sample.

At the concentration of 1000 μg/mL of kanamycin, the expression level of β15 and β39 transcripts are 168% and 17% of the wild-type transcripts (βN.1000), respectively. Although the expression level of β15 transcripts is much higher at these conditions, kanamycin does not seem to be responsible for this increase. The standard deviation is

too high to make any assumptions. As seen before, β39 transcripts do not seem to have suffered any modification on their transcription level.

Unpaired Student's t test revealed that there were no statistically significant p-values, which indicates that the used concentrations of either gentamicin or kanamycin treatments do not have a major role on the β39 transcripts. Factors like the identity of the termination codon and the surrounding mRNA sequence may be responsible for these results (Lee *et al*., 2012; Dabrowski *et al*., 2015).

However, a concentration of 1000 μg/mL of gentamicin revealed to be sufficient to modestly increase the expression level of β39 transcripts. Unfortunately, the standard deviation values were too high. The time of drug treatment should be increased, as well. As mentioned earlier, a different cell line could also be used (*e.g.* MEL cell line). The β-globin gene and its variants could also be cloned in a plasmid under control of a strong promoter.

5. Conclusion and Future Perspectives

The mechanisms of regulation of gene expression and mRNA-surveillance are crucial to the normal cell function. Several of these mechanisms are interconnected and interdependent.

Some mutations may lead to atypical phenotypes, and even cell death. Nonsense mutations are a type of point mutations characterized by an alteration in the gene sequence, in which a non-stop codon turns into a PTC. This may lead to the formation of truncated proteins. Nonsense mediated mRNA decay is an mRNA-surveillance mechanism responsible for the rapid degradation of aberrant transcripts, like PTC-containing mRNA.

It has been shown that some PTC-containing mRNA molecules do not go through nonsense mediated mRNA decay and may produce proteins that are toxic to the cells, showing clinical phenotypes. β-thalassemia is a heterogeneous group of diseases that, among other reasons, can be caused by nonsense mutations in the β-globin gene. β39 transcripts were shown to go through NMD and suffer rapid decay, however β15 transcripts do not go through NMD and a truncated protein is synthetized.

Several aminoglycosides were shown to enhance the competition between near cognate aminoacyl tRNAs and translation release factors, that are also directly linked to NMD, allowing the synthesis of full-length protein in DMD and cystic fibrosis patients.

In this project it was pretended to use gentamicin and kanamycin as readthrough compounds in order to produce full-length β-globin. Their effect should have been studied at the mRNA level and at the protein level, however, as βN-globin protein could not be properly detected by Western blot, it was decided that the effect of aminoglycosides would only be studied at the mRNA level.

Different concentrations of both aminoglycosides were used, however, results show that gentamicin and kanamycin do not have a significant effect on β15 and β39 mRNA levels.

Different assays evaluating the effect of the identity of the termination codon or the surrounding mRNA sequence context should be carried out in future experiments. Different drug concentrations or exposure times and different aminoglycosides should also be used in future assays.

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