Published in final edited form as:

Biotechnol Appl Biochem. ; 54(1): 41–52. doi:10.1042/BA20080266.

Development of K562 cell clones expressing β -globin mRNA carrying the β^039 thalassaemia mutation for the screening of correctors of stop-codon mutations

Francesca Salvatori*, Vera Cantale*, Giulia Breveglieri*, Cristina Zuccato†, Alessia Finotti†, Nicoletta Bianchi†, Monica Borgatti†, Giordana Feriotto†, Federica Destro†, Alessandro Canella†, Laura Breda‡, Stefano Rivella‡, and Roberto Gambari*,†,1

*BioPharmaNet, Department of Biochemistry and Molecular Biology, Ferrara University, Via Fossato di Mortara 74, 44100 Ferrara, Italy

†Laboratory for the Development of Pharmacological and Pharmacogenomic Therapy of Thalassaemia, Biotechnology Center, Ferrara University, Via Fossato di Mortara 64/b, 44100 Ferrara, Italy

‡Department of Pediatric Hematology–Oncology, Children’s Cancer and Blood Foundation Laboratories, Weill Medical College of Cornell University, New York, NY 10021, U.S.A

Abstract

Nonsense mutations, giving rise to UAA, UGA and UAG stop codons within the coding region of mRNAs, promote premature translational termination and are the leading cause of approx. 30 % of inherited diseases, including cystic fibrosis, Duchenne muscular dystrophy and thalassaemia. For instance, in β^039 -thalassaemia the CAG (glutamine) codon is mutated to the UAG stop codon, leading to premature translation termination and to mRNA destabilization through the well-described NMD (nonsense-mediated mRNA decay). In order to develop an approach facilitating translation and, therefore, protection from NMD, aminoglycoside antibiotics have been tested on mRNAs carrying premature stop codons. These drugs decrease the accuracy in the codon–anticodon base-pairing, inducing a ribosomal read-through of the premature termination codons. Interestingly, recent papers have described drugs designed and produced for suppressing premature translational termination, inducing a ribosomal read-through of premature but not normal termination codons. These findings have introduced new hopes for the development of a pharmacological approach to the therapy of β^039 -thalassaemia. In this context, we started the development of a cellular model of the β^039 -thalassaemia mutation that could be used for the screening of a high number of aminoglycosides and analogous molecules. To this aim, we produced a lentiviral construct containing the β^039 -thalassaemia globin gene under a minimal LCR (locus control region) control and used this construct for the transduction of K562 cells, subsequently subcloned, with the purpose to obtain several K562 clones with different integration copies of the construct. These clones were then treated with Geneticin (also known as G418) and other aminoglycosides and the production of β -globin was analysed by FACS analysis. The results obtained suggest that this experimental system is suitable for the characterization of correction of the β^039 -globin mutation causing β -thalassaemia.

Keywords

aminoglycoside antibiotics; K562 cell; locus control region; nonsense mutation; thalassaemia

Introduction

Nonsense mutations, giving rise to UAA, UGA and UAG stop codons within the coding region of mRNAs, promote premature translational termination [1–4] and are the leading cause of up to 30 % of inherited diseases [2,4], including cystic fibrosis [5], Duchenne muscular dystrophy [6] and thalassaemia [7–12]. For instance, in β^039 -thalassaemia the CAG (glutamine) codon of the β -globin mRNA is mutated to the UAG stop codon [7,8], leading to premature translation termination and to mRNA destabilization through the well-described NMD (nonsense-mediated mRNA decay) [3,4]. Other examples of stop mutations of the β -globin mRNA occur at positions 15 [9], 37 [10,11] and 127 [12] of the mRNA sequence.

In order to develop an approach facilitating translation and, therefore, protection from NMD, aminoglycoside antibiotics have been tested on mRNAs carrying premature stop codons. These drugs bind the decoding centre of the ribosome and decrease the accuracy in the codon–anticodon base-pairing, inducing a ribosomal read-through of premature termination codons [13,14]. Despite their correctional potential, long-term use of aminoglycoside antibiotics is associated with nephrotoxicity and ototoxicity [13–16]. Interestingly, recent papers have described safer drugs, without structural similarity to aminoglycosides, designed and produced for suppressing premature translational termination, inducing a ribosomal read-through of premature but not normal termination codons [1,5,17]. This could be the basis for novel therapeutic approaches able to correct at the mRNA translational level the genetic defects introducing stop codons.

Accordingly, the development of experimental model systems useful for the screening of this class of molecules is of great interest for the possible identification of drugs to be employed for the experimental therapy of diseases caused by stop codons.

As far as studies on erythropoiesis, the K562 cell line [18] is well known as a useful experimental model system to study the expression of embryo-fetal globin genes, as well as their modulation [19–23]. Interestingly, especially for reaching the aims of the present study, K562 cells express the β -globin gene at very low levels both in their uninduced state as well as after erythroid differentiation stimulated by a variety of chemical inducers, such as cytosine arabinoside [24], mithramycin [25,26] and rapamycin [27,28].

In the present paper, we report the development of K562 cell lines integrating multiple copies of the human β -globin gene carrying the β^039 -thalassaemia mutation. To this aim, we have (i) produced a lentiviral construct containing the β^039 -thalassaemia globin gene under a minimal LCR (locus control region) control; (ii) produced several K562 cell clones with different integration copies of the normal β wt-globin (where wt is wild-type) or the β^039 -thalassaemia constructs; (iii) analysed by quantitative real-time RT–PCR (reverse transcription–PCR) these clones for accumulation of the β wt-globin and β^039 -globin mRNAs; (iv) treated these clones with Geneticin; (v) analysed the production of β -globin by FACS analysis; (vi) treated the best clone carrying β^039 -globin genes and expressing the β^039 -globin mRNA with Geneticin and other aminoglycosides in order to verify whether these experimental cellular systems are suitable for the screening of correctors of point mutations.

Materials and methods

Site-directed mutagenesis

Mutagenesis was performed by using the QuikChange® II Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) in order to introduce the β^039 -thalassaemic point mutation into the human β -globin gene in the pSL1180DT+ β vector. A couple of mutant primers were designed (β^039 forward primer, 5'-GGT CTA CCC TTG GAC CTA GAG GTT CTT TGA GTC-3'; β^039 reverse primer, 5'-GAC TCA AAG AAC CTC TAG GTC CAA GGG TAG ACC-3'), having complementary sequences and containing the desired mutated nucleotide (indicated in bold). The mutagenesis reaction was performed in a final volume of 25 μ l, containing 25 ng of pSL1180DT+ β plasmid template, 1 $\times$ reaction buffer [20 mM Tris/HCl, pH 8.8, 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂ SO₄, 0.1 mg/ml BSA and 0.1 % Triton X-100], 0.5 μ l of dNTP mix and 62.5 ng of mutagenesis primers, by using 1.25 units of PfuUltra HF DNA polymerase. The thermal reaction was performed by using the GeneAmp PCR System 9600 (PerkinElmer, Waltham, MA, U.S.A.): after a first denaturation at 94 °C for 3 min, 22 cycles were performed, consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and elongation at 68 °C for 8 min. At the end of the mutagenesis reaction, the amplification products were digested with 5 units of the restriction endonuclease DpnI, at 37 °C for 1 h, so as to remove the parental not mutated DNA. A 5 μ l portion of the digestion reaction was used to transform 120 μ l of ultracompetent *Escherichia coli* JM109 bacteria: DNA and bacteria were incubated on ice for 4 h; then, after a thermic shock at 42 °C for 45 s and immediately keeping on ice for 2 min, 1 ml of LB (Luria–Bertani) medium (10 g/l bacto-tryptone, 5 g/l yeast extract and 10 g/l NaCl) was added and an incubation at 37 °C for 1 h under slow agitation was performed; finally, bacteria were plated on Petri plates containing semisolid medium (LB medium with 15 g/l bacto-agar) in the presence of 100 μ g/ml ampicillin, and incubated at 37 °C overnight. The bacterial clones obtained were screened for the incorporation of the recombinant plasmid construct, whose nucleotide sequence was finally confirmed by DNA sequencing.

Heat lysis of bacterial clones

In order to rapidly obtain a plasmid template to be amplified by PCR, the bacterial clone was washed twice, then suspended in 20 μ l of ultrapure water, lysed at 96 °C for 6 min and immediately chilled on ice. After centrifugation at a maximum speed (15 000 g) for 20 min at 4 °C, the incorporated plasmid was collected in the supernatant.

DNA sequencing

In each PCR reaction, 2 μ l of supernatant obtained after heat lysis of bacterial clones was amplified by Taq DNA polymerase using the BGF1 (5'-AGA CCT CAC CCT GTG GAG CC-3') and BGR1 (5'-AGT TCT CAG GAT CCA CGT GCA-3') primers, which amplify exons 1 and 2 of the β -globin gene leading to a 601 bp PCR product (Figure 2). PCR was performed in a final volume of 100 μ l, containing 50 mM KCl, 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl₂, 0.1 % Triton X-100, 33 μ M dNTPs and 0.33 μ M PCR primers, by using 2 units per reaction of Taq DNA polymerase. The 35 PCR cycles were as follows: denaturation, 30 s, 95 °C; annealing, 20 s, 66 °C; and elongation, 2 min, 72 °C. The β -globin PCR products were purified with Microcon®-100 (Millipore, Billerica, MA, U.S.A.) and sequenced using the ABI PRISM™ BigDye™ Terminator v 1.1 Cycle Sequencing Ready Reaction kit and the ABI Prism™ 377 DNA sequencer (Applied Biosystems, Warrington, Cheshire, U.K.).

Virus production

Viral stocks were generated by transfection of pCCL. β wt.PGW or pCCL. β^0 39.PGW and the helper vectors pMD2.VSVG [coding for the envelope protein VSV-G (vesicular-stomatitis-virus glycoprotein)], pMDLpg.RRE [coding for the proteins GAG (group antigens polypeptide) and the reverse transcriptase POL] and pRSV.REV [coding for the protein REV (regulator of expression of viral proteins)] [29] into HEK-293T cells [HEK-293 cells (human embryonic kidney cells) expressing the large T-antigen of SV40 (simian virus 40)]. The HEK-293T (5×10^6) cells were plated in 10-cm-diameter cell-culture dishes 24 h before transfection in DMEM (Dulbecco's modified Eagle's medium; Cambrex-Biowhittaker Europe, Milan, Italy) with 10 % (v/v) FBS (fetal bovine serum; Biowest, Nuaillé, France), 2 mM L-glutamine (Cambrex-Biowhittaker), 100 units/ml penicillin and 100 μ g/ml streptomycin (Pen-Strep; Cambrex-Biowhittaker) in a humidified atmosphere of 5 % CO₂/air. The culture medium was changed to IMDM (Iscove's modified Dulbecco's medium; Cambrex-Biowhittaker) 2 h before transfection. A total of 25.5 μ g of DNA was used for transfection of one dish: 3 μ g of the envelope plasmid pMD2.VSVG, 5 and 2.5 μ g of the two packaging plasmids, pMDLpg.RRE and pRSV.REV respectively and 15 μ g of the lentiviral vector. A 0.1 $\times$ TE (10 mM Tris, pH 8.0, plus 1 mM EDTA) and water solution (2:1) was added to the plasmid up to a final volume of 450 μ l. The precipitate was finally formed after adding 50 μ l of 2.5 mM CaCl₂ solution, mixing well and adding dropwise 500 μ l of 2 $\times$ Hepes-buffered saline (281 mM NaCl, 100 mM Hepes and 1.5 mM Na₂ HPO₄, pH 7.12) while vortex-mixing. The precipitate was immediately added to the cultures. The medium was replaced after 16 h; the viral supernatant was collected after another 24 h, replaced and again collected after 24 h. Each time, the supernatant was filtered through 0.2 μ m pore size cellulose acetate filters, divided into aliquots and frozen. The viral titre was calculated as previously described [30].

Human K562 cell cultures and K562 cell clones carrying the β wt-globin or the β^0 39-globin gene

The human leukaemia K562 cells [14] were cultured in a humidified atmosphere of 5 % CO₂/air in RPMI 1640 medium (Sigma, St Louis, MO, U.S.A.) supplemented with 10 % (v/v) FBS (Biowest), 2 mM L-glutamine (Cambrex-Biowhittaker), 100 units/ml penicillin and 100 μ g/ml streptomycin (Cambrex-Biowhittaker). Cell growth was studied by determining the cell number per ml with a ZF Coulter counter (Coulter Electronics, Hialeah, FL, U.S.A.). Two lentiviral constructs were used to generate stable K562 clones integrating human normal and β^0 39-globin genes. The pCCL. β PGW lentiviral construct carries two LTR (long terminal repeat) sequences, the SV40 origin of replication, a GFP (green fluorescent protein) gene under the control of the PGK (phosphoglycerate kinase) promoter, and the β -globin gene, under the control of the β -globin gene promoter and a minimal LCR of the human β -like globin gene cluster. The second construct was developed replacing the wild-type β -globin gene with a β^0 39-globin gene produced by site-directed mutagenesis. Transduction was carried out by plating 10^6 K562 cells in 9.5 cm² dishes with 45 % RPMI 1640 (Sigma) and 45 % IMDM (Cambrex-Biowhittaker), 10 % FBS (Biowest), 2 mM L-glutamine (Cambrex-Biowhittaker), 100 units/ml penicillin and 100 μ g/ml streptomycin (Pen-Strep; Cambrex-Biowhittaker) at 37 °C in a humidified atmosphere of 5 % CO₂/air. Cells were infected with the thawed viral supernatant at various MOI (multiplicity of infection) values (MOI = 2–4). In order to facilitate the cell infection, 10 μ l of the 800 μ g/ μ l transduction agent polybrene (Chemicon International, Millipore) was added to the plated K562 cells, which were subsequently cultured in a 5 % CO₂ incubator. After 7 days, K562 cells were cloned by limiting dilutions and GFP-producing clones were identified under a fluorescence microscope and further characterized. Treatments with aminoglycoside antibiotics were carried out by adding the appropriate drug concentrations to the cells, seeding at 30 000 or 8000 cells/ml and incubating at 37 °C in a humidified atmosphere of 5 % CO₂/air for 3–5

days. The medium was not changed during the treatment period. Geneticin was from Gibco (Invitrogen-Life Technologies, Carlsbad, CA, U.S.A.), gentamicin, streptomycin, tobramycin and amikacin from Sigma.

Real-time quantitative PCR

To quantify the amount of β -globin gene sequences in the K562 genomic DNA from β wt-globin and β^{039} -globin-K562 cell clones, we used 100–200 ng of template DNA and a standard curve in the range 50–300 ng of K562 genomic DNA as the control. The β -globin gene sequences in β wt-globin and β^{039} -globin-K562 cell clones were quantified with respect to the γ -globin target gene, using the same amount of genomic DNA. The γ -globin gene represents a good reference because of its location in the same chromosome, within the same gene cluster, of the β -globin gene. The fold of transduced β -globin genes was expressed as $2^{-\Delta\Delta C_t}$, where C_t represents the threshold cycle value and $\Delta\Delta C_t$ represents the subtraction of the ΔC_t obtained using genomic DNA from untransduced K562 cells (the original K562 cell line) from the ΔC_t derived from β wt-globin and β^{039} -globin-K562 cell clones. ΔC_t is the difference between the amplification cycle equivalent to a significant fluorescence level (C_t) obtained by the amplification of the β -globin gene and the corresponding amplification cycle of the γ -globin gene. This equation can be applied to have a relative quantification of our templates because the slopes of β -globin and γ -globin gene amplifications are comparable. PCR primers and probes were: β -globin forward primer, 5'-CAA GAA AGT GCT CGG TGC CT-3'; β -globin reverse primer, 5'-GCA AAG GTG CCC TTG AGG T-3'; β -globin probe, 5'-FAM-TAG TGA TGG CCT GGC TCA CCT GGA C-TAMRA-3'. The fluorescent reporter and the quencher were: FAM (6-carboxyfluorescein) and TAMRA (6-carboxytetramethylrhodamine) respectively. For real-time PCR analysis we used as a reference gene the γ -globin gene. PCR primers and probe were: γ -globin forward primer, 5'-TGG CAA GAA GGT GCT GAC TTC-3'; γ -globin reverse primer, 5'-TCA CTC AGC TGG GCA AAG G-3'; γ -globin probe, 5'-FAM-TGG GAG ATG CCA TAA AGC ACC TGG-TAMRA-3'. The probe was fluorescently labelled with FAM (Applied Biosystems). Quantitative real-time PCR was carried out using a 7700 Sequence Detection System version 1.7 (Applied Biosystems).

RNA isolation and RT-PCR analysis

K562 cell clones were collected by centrifugation at 400 *g* for 5 min at 4 °C, washed with PBS and lysed in 1 ml of TRIzol[®] reagent (Gibco–Invitrogen–Life Technologies), according to the manufacturer's instructions. The isolated RNA was washed once with cold 75 % ethanol, dried and dissolved in diethylpyrocarbonate-treated water before use. For gene expression analysis 1 μ g of total RNA was reverse transcribed by using random hexamers. To quantify the amount of β -globin mRNA we used 20 ng of cDNA and a standard curve of normal control DNA in the range 5–60 ng. Quantitative real-time PCR was carried out using a Custom TaqMan[®] SNP Genotyping Assays kit (Applied Biosystems) in a 7700 Sequence Detection System version 1.7 (Applied Biosystems). PCR was performed in a final volume of 25 μ l, containing 12.5 μ l of TaqMan[®] Universal PCR Master Mix and 0.625 μ l of 40 $\times$ assay mix. After two initial steps, 2 min at 50 °C and 10 min at 95 °C, the 40 cycles were as follows: 15 s at 92 °C and 60 s at 60 °C. PCR primers and probes were: primer forward, 5'-CAG GCT GCT GGT GGT CTA C-3'; primer reverse, 5'-AGT GGA CAG ATC CCC AAA GGA-3'; probe β wt, 5'-VIC-AAA GAA CCT CTG GGT CCA-TAMRA; probe β^{039} , 5'-FAM-CAA AGA ACC TCT AGG TCC A-TAMRA-3'. The probes β wt and β^{039} were fluorescently labelled with VIC and FAM (Applied Biosystems) respectively to quantify the β wt and β^{039} -globin mRNA in a single reaction. For real-time PCR analysis we always used as a reference gene the endogenous control human GAPDH kit (Applied Biosystems), in the gene expression assay. The fluorescent reporter and the quencher of the GAPDH probe were: VIC and TAMRA respectively.

FACS analysis

K562 cells treated with Geneticin were permeabilized and marked with the antibody against β -globin using the Cytotfix/Cytoperm™ kit (BD Biosciences Pharmingen, Franklin Lakes, NJ, U.S.A.). A total of 10^6 cells were first washed with 500 μ l of PBS and then incubated with 500 μ l of BD Cytotfix-Cytoperm solution for 20 min at 4 °C, in order to permit the cellular permeabilization. After the incubation the cells were washed twice and incubated with 300 μ l of 1 $\times$ PBS + 1 % BSA (Sigma) solution for 1 h at room temperature (25 °C) in darkness. The BSA has the capacity to block the aspecific binding sites. The cells were then collected by centrifugation and incubated with 30 μ l of β -globin-PE (phycoerythrin) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), diluted 1:10 in 1 $\times$ PBS + 1 % BSA over night at 4 °C in darkness. After the incubation, the cells were washed with 500 μ l of 1 $\times$ PBS and resuspended with 30 μ l of 1 $\times$ PBS. The cellular suspension was transferred to a FACS tube and 500 μ l of staining buffer (1 $\times$ PBS + 1 % FBS) were added. The analysis was performed with FACScan (flow-activated cell sorting; Becton Dickinson), using the Cell Quest Pro software (Becton Dickinson).

Results

Production of the lentiviral construct carrying the mutated human β^{039} -globin gene

Figure 1 shows the strategy followed to produce the lentiviral constructs used for generation of the K562 cell clones. We first introduced the β^{039} -thalassaemic point mutation into the human β -globin gene in the pSL1180DT+ β vector (depicted in the upper panel of Figure 1), by using a site-directed *in vitro* mutagenesis system (from Stratagene). Then the dTNS9- β^{039} was produced as described in Figure 1 and characterized by restriction enzyme digestion as reported in Figure 2(A). Finally, the pCCL. β^{039} .PGW vector was generated by replacing the ClaI–XbaI fragment of the pCCL. β .PGW with the ClaI–XbaI fragment of the dTNS9- β^{039} construct. The obtained pCCL. β^{039} .PGW construct, characterized by PCR amplification and direct sequencing as reported in Figure 2(B), displays two LTR sequences, the SV40 origin of replication, a GFP gene under the control of the PGK promoter, the β -globin gene, under the control of the β -globin gene promoter, and a minimal LCR of the human β -like globin gene cluster (Figure 1). The presence of the PGK–GFP gene allows the high-throughput screening of cells integrating this construct, giving at the same time some preliminary information on the number of integration events.

Production of K562 cell clones expressing human β -globin genes carrying the β^{039} -thalassaemia mutation

The two constructs shown in Figure 1 have been employed in transduction experiments using K562 cells as the model system, and 13 clones have been identified and further characterized. Figure 3 shows the fluorescence intensities of eight of these clones (wt3, wt5, wt6 and wt7 carrying the normal β -globin gene, whereas m1, m4, m5 and m6 carry the mutated β^{039} -globin gene). As it is clearly evident, clones wt3 and m6 display the highest levels of fluorescence, suggesting the integration of high copy numbers of the respective constructs.

Analysis of the integration units of the β wt-globin and the β^{039} -globin genes

The analysis of the integration units of the β wt-globin-K562 and β^{039} -globin-K562 cells was performed by PCR using genomic DNA isolated from the different clones and performing real-time PCR (a representative PCR experiment is shown in Figures 4A and 4B). The results obtained are shown in Figures 4(C) and 4(D), and clearly indicate that higher numbers of integrated constructs are present in wt1, wt2, wt4, wt5, wt6, wt7 (β wt-globin-K562 cells) and m1, m2, m3 (β^{039} -globin-K562 cells) clones. The highest numbers

of integrated constructs (transgene fold ranging from 5 to 10) are present in wt3 (β wt-globin-K562 cells) and m4, m5 and m6 (β^0 39-globin-K562 cells) clones. This analysis has been carried out three times with similar results. Interestingly, when FACS analysis was performed on all of the clones, we noted that there is a very good correlation between the GFP median fluorescence intensity (Figures 4C and 4D, black histograms) and the number of the integrated constructs (Figures 4C and 4D, open histograms). This is not surprising, since the GFP gene is under the control of the constitutive PGK promoter. On the contrary, we cannot exclude that the site(s) of integration might influence (either positively or negatively) the transcription of the β wt and β^0 39-globin genes, which are under the control of the β -globin promoter and a minimal LCR sequence (see Figure 1). Furthermore, we are expecting the well-known phenomenon of NMD [10,11] of the β^0 39-globin mRNA in the β^0 39-globin-K562 cell clones. Therefore a careful analysis of the mRNA levels was performed by quantitative real-time RT-PCR, as described in detail elsewhere [22–28].

Accumulation of the β -globin and β^0 39-globin mRNAs in β wt-globin-K562 and β^0 39-globin-K562 cell clones

The extent of accumulation of β -globin and β^0 39-globin mRNAs in the β wt-globin-K562 and β^0 39-globin-K562 cell clones was analysed by RT-PCR using as the template RNA isolated from exponentially growing cells. We were interested in identifying β wt-globin-K562 and β^0 39-globin-K562 cell clones accumulating similar amounts of β -globin mRNA sequences. The results obtained gave clear evidence that within the β wt-globin-K562 clones, high expression (relative to wt1) was detected in wt6 and low expression in wt3, wt5 and wt7 clones (Figure 5A); on the other hand, within the β^0 39-globin-K562 clones, the β -globin gene was highly expressed (in comparison with m3) in K562 cell clones m6, m5, m4 and m1 and poorly expressed in K562 cell clone m2 (Figure 5B). Despite the fact that no silencing effect was noted, no clear relationship is evident between levels of gene expression and number of integration units. This is however as expected, since transcription might depend also on the site of integration. Moreover, in β^0 39-globin-K562 cell clones the accumulation of β -globin mRNA sequences is affected by the known effect of NMD [3,4]. After comparative analysis of the RT-PCR data, clones wt3 (β wt-globin-K562 cells) and m5 (β^0 39-globin-K562 cells) were selected for the experiments employing possible correction with Geneticin, due to the fact that, among the β wt-globin and β^0 39-globin-K562 clone sets, they express similar, although not identical, levels of β -globin mRNA molecules, as depicted in Figures 5(C) and 5(D). Interestingly, clone m5 displays higher β -globin gene integration units and GFP production with respect to clone wt3, suggesting that, as expected, NMD affects β^0 39-globin mRNA. The differential expression of β -globin mRNA between m5 and wt3 is maintained when the culture conditions were changed from expansion to differentiation, by treating cells with cytosine arabinoside (F. Salvatori, G. Breveglieri, I. Lampronti, C. Zuccato, N. Bianchi, M. Borgatti and R. Gambari, unpublished work).

Effects of Geneticin on β -globin production in the m5 clone

The effects of Geneticin on the β -globin production were analysed after FACS analysis of untreated compared with Geneticin-treated cells. As expected, very low levels of β -globin are detected in control K562 cells (Table 1, left column). It is well known indeed that K562 cells are committed to γ -globin gene expression and produce only very low levels of β -globin mRNA. In order to verify the effects of Geneticin on β -globin production in the wt3 β wt-globin-K562 cell clone, cells were, in a first experiment, cultured in the absence or presence of 400 ng/ μ l Geneticin. At the end of the treatment, cells were recovered and labelled with the PE-labelled β -globin monoclonal antibody. This labelling allows discrimination, under FACS analysis, of the green fluorescence of GFP from the red fluorescence of β -globin-PE monoclonal antibody. When FACS analysis was performed (Figures 6A and 6B) it was clearly evident that Geneticin did not induce major changes in β -

globin production in wt3 β wt-globin-K562 cells. By sharp contrast, when the same experiment was carried out using the m5 β^{039} -globin K562 cell clone, a clear increase in cells producing β -globin was detected (Figures 6C and 6D). No major changes in GFP production were observed after treatment with Geneticin of both wt3 β wt-globin-K562 and m5 β^{039} -globin-K562 cell clones. The results of three independent experiments employing increasing concentrations of Geneticin are shown in Figure 7(A), confirming a concentration-dependent increase in β -globin-containing cells when the m5 β^{039} -globin-K562 cell clone is treated with Geneticin. The effect of Geneticin was found to be higher than that displayed by gentamicin (Figure 7B).

Finally, Table 1 shows a summary of the comparative FACS analysis of the m5 β^{039} -globin K562 cell clone treated with the aminoglycosides Geneticin, gentamicin, streptomycin, amikacin and tobramycin. As it is evident, gentamicin and streptomycin were found to be less efficient than Geneticin. Among the tested aminoglycosides, tobramycin and amikacin were found to be almost ineffective. These results support the concept that our cellular screening system could be useful in ranking the activity of correctors of stop mutations. As expected, and shown in Table 1 (left and middle columns), no significant increase in β -globin-positive cells was observed when the treatment was performed on original K562 cells (expressing very low amounts of β -globin), as well as on the wt3 β wt-globin-K562 cell clone (expressing a high amount of β -globin). In addition, Table 1 indicates that the FACS data concerning untreated m5 cells are very similar to those found with the original K562 cells, suggesting that the β^{039} -globin mRNA accumulated by m5 cells is not translated, unless the m5 cells are treated with aminoglycosides. Taken together, the results obtained suggest that the effects of aminoglycosides are related to a read-through effect, rather than an induction of endogenous or *de novo* integrated wild-type β -globin genes.

Discussion

In the present paper, we described the development of cellular K562 cell systems carrying β -globin genes with a stop mutation causing β^0 -thalassaemia. These K562 clones were generated by stable transduction with a lentiviral vector carrying wild-type or mutated β^{039} -globin genes under the control of a minimal LCR region. These clones express the GFP protein at a level correlated with the integrated copy number, whereas in several clones a direct relationship between copy number and accumulation of the β wt or β^{039} -globin mRNA was not evident, possibly due to the site of integration, which might affect the tissue-specific expression of the β -globin gene, independently of the presence of the stop mutation.

As a proof of principle of the possible application of these K562 cell clones, Geneticin was used. Geneticin, indeed, has been reported to retain the very interesting property to induce read-through of mRNAs carrying translation stop mutations leading to production of truncated and functionally inactive proteins [31,32]. The results obtained suggest that this experimental system is suitable for the characterization of correctors of the β^{039} -globin mutation causing β -thalassaemia. This was reproducibly obtained using Geneticin on K562 cell clones carrying β^{039} -globin genes. In addition, our experimental system allows ranking of the activity of several other aminoglycosides, such as gentamicin, streptomycin, tobramycin and amikacin, as we have demonstrated in Figure 7 and Table 1. No major effects on β -globin synthesis were found on the original K562 and on the K562- β -wt3 clones (Table 1).

Accordingly, the development of experimental model systems useful for the screening of this class of molecules is of great interest for the possible identification of drugs to be employed for the experimental therapy of diseases caused by stop codons. K562 clones integrating the human β^{039} -globin gene appear to be a low-throughput cellular system for

the characterization of molecules selected by means of high-throughput strategies, such as those employing reporter genes carrying stop mutations mimicking molecular defects leading to human pathologies.

In this respect, we have to point out that several human pathologies caused by stop mutations are known, including cystic fibrosis [5], muscular dystrophy [6] and the Hurler syndrome [13]. Accordingly, several research groups have started projects aimed at developing molecules able to promote read-through, to be tested on *in vitro* and *in vivo* model systems. For instance, in a recent paper by Welch et al. [1], two high-throughput screens (comprising 800 000 low-molecular-mass compounds) were performed to identify compounds that promoted UGA nonsense suppression. Chemical scaffolds were identified and optimized through extensive medicinal chemistry efforts. These analyses identified PTC124 {3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]benzoic acid; C₁₅ H₉FN₂ O₃} as a candidate for further development. The selectivity of PTC124 for premature termination codons, its well-characterized activity profile, oral bioavailability and pharmacological properties indicate that this drug may have broad clinical potential for the treatment of a large group of genetic disorders with limited or no therapeutic options.

The results shown in the present paper allow us to propose our developed K562 cell lines as a useful cellular biosensor to verify whether compounds suppressing the effects of premature termination codons might be of interest for the possible development of drugs of interest for thalassaemia.

Acknowledgments

We thank Dr Mirko Pinotti (Department of Biochemistry and Molecular Biology, Ferrara University, Ferrara, Italy) for helpful suggestions.

Funding

This work was supported by the Fondazione Cariparo (Cassa di Risparmio di Padova e Rovigo) (grant to R.G.); the Associazione Italiana per la Ricerca sul Cancro (AIRC) (grant to R.G.); Programmi di Ricerca Cofinanziati-2005 (Cofin-2005) [grant number 2005038704-005 (to R.G.)]; the University of Ferrara STAMINA Project (grant to R.G.); the eInfrastructure for the Thalassaemia Research Network (UE ITHANET) Project; Telethon [grant number GGP07257 (to R.G.)]; the Regione Emilia-Romagna Spinner Project; and by the Associazione Veneta per la Lotta alla Talassemia (AVLT), Rovigo, Italy.

Abbreviations used

C_t	threshold cycle value
FAM	6-carboxyfluorescein
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
LB	Luria–Bertani
LCR	locus control region
LTR	long terminal repeat
MOI	multiplicity of infection
NMD	nonsense-mediated mRNA decay
PE	phycoerythrin

PGK	phosphoglycerate kinase
RT-PCR	reverse transcription-PCR
SV40	simian virus 40
TAMRA	6-carboxytetramethylrhodamine
wt	wild-type

References

1. Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, Paushkin S, Patel M, Trotta CR, Hwang S, et al. *Nature*. 2007; 447:87–91. [PubMed: 17450125]
2. Mendell JT, Dietz HC. *Cell*. 2001; 107:411–414. [PubMed: 11719181]
3. Holbrook JA, Neu-Yilik G, Hentze MW, Kulozik AE. *Nat Genet*. 2004; 36:801–808. [PubMed: 15284851]
4. Stalder L, Mühlemann O. *Trends Cell Biol*. 2008; 18:315–321. [PubMed: 18524595]
5. Du M, Liu X, Welch EM, Hirawat S, Peltz SW, Bedwell DM. *Proc Natl Acad Sci USA*. 2008; 105:2064–2069. [PubMed: 18272502]
6. Kuehn BM. *JAMA, J Am Med Assoc*. 2007; 298:1385–1386.
7. Trecartin R, Liebhaver SA, Chang JC, Lee KY, Kan YW, Furbetta M, Angius A, Cao A. *J Clin Invest*. 1981; 68:1012–1017. [PubMed: 6457059]
8. Piras I, Vona G, Falchi A, Latini V, Ristaldi S, Vacca L, Varesi L, Calo CM. *Am J Hum Biol*. 2005; 17:765–772. [PubMed: 16254903]
9. Ahmed M, Stuhmann M, Bashawri L, Kühnau W, El-Harith EH. *Ann Hematol*. 2001; 80:629–633. [PubMed: 11757720]
10. Kornblit B, Taaning P, Birgens H. *Hemoglobin*. 2005; 29:209–213. [PubMed: 16114184]
11. Li D, Liao C, Li J, Tang X. *Hemoglobin*. 2006; 30:171–173. [PubMed: 16798641]
12. Préhu C, Pissard S, Al-Sheikh M, Le Niger C, Bachir D, Galactéros F, Wajcman H. *Hemoglobin*. 2005; 29:229–233. [PubMed: 16114188]
13. Keeling KM, Brooks DA, Hopwood JJ, Li P, Thompson JN, Bedwell DM. *Hum Mol Genet*. 2001; 10:291–299. [PubMed: 11159948]
14. Kaufman RJ. *J Clin Invest*. 1999; 104:367–368. [PubMed: 10449426]
15. Ogle JM, Brodersen DE, Clemons WM, Tarry MJ, Carter AP, Ramakrishnan V. *Science*. 2001; 292:897–902. [PubMed: 11340196]
16. Zingman LV, Park S, Olson TM, Alekseen AE, Terzic A. *Clin Pharm Ther*. 2007; 81:99–103.
17. Hirawat S, Welch EM, Elfring GL, Northcutt VJ, Paushkin S, Hwang S, Leonard EM, Almstead NG, Ju W, Peltz SW, Miller LL. *J Clin Pharmacol*. 2007; 47:430–444. [PubMed: 17389552]
18. Luzzio CB, Luzzio BB. *Blood*. 1975; 45:321–334. [PubMed: 163658]
19. Rutherford TR, Clegg JB, Weatherall DJ. *Nature*. 1979; 280:164–165. [PubMed: 95354]
20. Rutherford TR, Clegg JB, Higgs DR, Jones RW, Thompson J, Weatherall DJ. *Proc Natl Acad Sci USA*. 1981; 78:348–352. [PubMed: 6264439]
21. Osti F, Corradini FG, Hanau S, Matteuzzi M, Gambari R. *Haematologica*. 1997; 82:395–401. [PubMed: 9299849]
22. Bianchi N, Ongaro F, Chiarabelli C, Gualandi L, Mischiati C, Bergamini P, Gambari R. *Biochem Pharmacol*. 2000; 60:31–40. [PubMed: 10807942]
23. Bianchi N, Chiarabelli C, Borgatti M, Mischiati C, Fibach E, Gambari R. *Br J Haematol*. 2001; 113:951–961. [PubMed: 11442489]
24. Cortesi R, Gui V, Gambari R, Nastruzzi C. *Am J Hematol*. 1999; 62:33–43. [PubMed: 10467274]
25. Bianchi N, Osti F, Rutigliano C, Corradini FG, Borsetti E, Tomassetti M, Mischiati C, Feriotto G, Gambari R. *Br J Haematol*. 1999; 104:258–265. [PubMed: 10050705]

26. Fibach E, Bianchi N, Borgatti M, Prus E, Gambari R. *Blood*. 2003; 102:1276–1281. [PubMed: 12738678]
27. Mischiati C, Sereni A, Lampronti I, Bianchi N, Borgatti M, Prus E, Fibach E, Gambari R. *Br J Haematol*. 2004; 126:612–621. [PubMed: 15287957]
28. Fibach E, Bianchi N, Borgatti M, Zuccato C, Finotti A, Lampronti I, Prus E, Mischiati C, Gambari R. *Eur J Haematol*. 2006; 77:437–441. [PubMed: 16939628]
29. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. *J Virol*. 1998; 72:8463–8471. [PubMed: 9765382]
30. Sastry L, Johnson T, Hobson MJ, Smucker B, Cornetta K. *Gene Ther*. 2002; 9:1155–1162. [PubMed: 12170379]
31. Sangkuhl K, Schulz A, Römler H, Yun J, Wess J, Schöneberg T. *Hum Mol Genet*. 2004; 3:893–903. [PubMed: 14998935]
32. Lai CH, Chun HH, Nahas SA, Mitui M, Gamo KM, Du L, Gatti RA. *Proc Natl Acad Sci USA*. 2004; 101:15676–15681. [PubMed: 15498871]

Figure 1.
Strategy followed to produce the lentiviral constructs used for generation of the K562 cell clones

Figure 2. Characterization of the dTNS9- β^{039} vector

(A) Electrophoretic analysis of the fragments obtained by digestion of dTNS9- β^{039} with ClaI and XbaI enzymes. M, molecular mass marker 1 kb DNA ladder (MBI Fermentas). (B) Characterization of the pCCL- β^{039} .PGW vector: electrophoretic analysis of the PCR product obtained by amplification of exons 1 and 2 of the β -globin gene and electropherogram achieved by sequencing the 601-bp PCR fragment. M, molecular mass marker pUC Mix Marker 8 (MBI Fermentas).

Figure 3. Fluorescence intensities of eight representatives of the 13 clones obtained, four containing the β wt-globin gene (upper four panels) and four containing the β^{039} -globin gene (lower four panels)

Figure 4. Analysis of the integration and GFP fluorescence of the β wt-globin and β^{039} -globin K562 cell clones

(A, B) Representative real-time PCR analysis of β -globin DNA from (A) wt3 (open circles), wt1 (open squares) clones and (B) m5 (black circles) and m3 (black squares) clones; the PCR probe used amplifies human β -globin gene sequences. (C, D) White bars, integrated β wt-globin and β^{039} -globin genes in the genome of the β wt-globin-K562 (C) and β^{039} -globin-K562 (D) cells. Black bars, GFP fluorescence. The results represent the means $\pm$ S.D. for three independent determinations. In order to determine the integration efficiency of the β wt-globin and β^{039} -globin genes by real-time PCR, the obtained C_t values were compared with those obtained using primers amplifying γ -globin gene sequences.

Figure 5. Expression of the β -globin gene in β^0 wt-globin and β^039 -globin K562 cell clones (A, B) RT-PCR analysis showing the β -globin mRNA content in β^0 wt-globin (A) and β^039 -globin-K562 (B) clones (means $\pm$ S.D. for three independent determinations). Results represent the fold change in β -globin mRNA content with respect to wt1 (A) and m3 (B). The relative content of β -globin mRNA of the original K562 cells is also shown (left side of each panel). (C, D) Profiles of quantitative RT-PCR analysis performed on RNA isolated from β^0 wt-globin-K562 clone wt3 (open symbols) and from β^039 -globin-K562 clone m5 (closed symbols) using primers amplifying GAPDH (C) and β -globin (D) mRNA sequences. Two different probes were used for the β -globin mRNA: a β^0 wt probe recognizing the β^0 wt-globin mRNA (open symbols) and a β^039 probe recognizing the β^039 -globin mRNA (closed symbols).

Figure 6. Effect of 400 ng/ μ l Geneticin on the production of β -globin and GFP in β wt-globin K562 clone wt3 (A, B) and β^{039} -globin-K562 clone m5 (C, D). FACS analysis of untreated (A, C) compared with Geneticin-treated (B, D) cells is shown.

Figure 7. Effect of increasing concentrations of Geneticin (A) and gentamicin (B) on the proportion (%) of β -globin-containing β^{39} -globin-K562 cells
FACS analyses were conducted as shown in Figure 6. The results are means $\pm$ S.D. for three independent determinations.

Table 1Induction of β -globin accumulation by aminoglycosides

Results are presented as a percentage of β-globin-containing cells.				
Treatment	Cell line ...	β-Globin-containing cells (%)		
		K562 (original)	wt3 βwt-globin-K562 cells	m5 $\beta^0$39-globin K562 cells
None		1.6 $\pm$ 0.3	73.7 $\pm$ 18.3	1.5 $\pm$ 0.4
Geneticin		1.5 $\pm$ 0.4	85.3 $\pm$ 4.5	51.5 $\pm$ 22.3
Gentamicin		1.8 $\pm$ 0.1	73.4 $\pm$ 7.5	5.65 $\pm$ 0.7
Streptomycin		1.4 $\pm$ 0.2	64.5 $\pm$ 11.4	3.74 $\pm$ 0.5
Tobramycin		1.1 $\pm$ 0.2	70.1 $\pm$ 6.8	2.2 $\pm$ 0.2
Amikacin		1.5 $\pm$ 0.4	67.3 $\pm$ 8.5	2.1 $\pm$ 0.5