

Sodium Butyrate and Valproic Acid as Splicing Restoring Agents in Erythroid Cells of β -Thalassemic Patients

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Background: β -Thalassemia is a common autosomal recessive disorder in human caused by a defect in β -globin chain synthesis. The most common mutations causing β -Thalassemia have been found to be splicing mutations. Most of which activate aberrant cryptic splicing/sites without complete disruption of normal splicing. IVSI-110 mutation, a common splicing mutation, leads to a 90% reduction of normal β -globin synthesis and lead to blood transfusion dependency in the homozygote forms. However, modulation of splicing can be achieved by activation or suppression of transacting factors such as SR (Serine, Arginine) amino acids and hnRNPs (Heterogeneous ribonucleoprotein particle) through drugs.

Objectives: The aim of this study was to investigate the effects of NaBu, isoBu and VPA drugs on restoration of splicing of IVSI-110 β -Thalassemia pre-mRNA in human.

Materials and Methods: Primary erythroid cells derived from IVSI-110 β -Thalassemia patients were cultured ex vivo and differentiated in the presence of 0.5 and 1 mM of Na-Butyrate (NaBu), 0.5 mM Isobutyramide (isoBu) and 100 μ M Valproic acid (VPA). RT-PCR analysis was used to evaluate the effect of the drugs in correction of normal splicing in β -globin mRNAs.

Results: Following treatment with NaBu, isoBu and VPA, the level of normal β -globin mRNA in Primary erythroid cells derived from IVSI-110 β -Thalassemia patients, increased 1.7, 1.5, 1.4 fold, respectively relative to normal β -globin mRNAs. Higher splicing restoration was achieved by NaBu, a histone deacetylase inhibitor, known to upregulate the expression of splicing factors.

Conclusions: The results highlighted the therapeutic potential of splicing modulation for genetic diseases caused by splicing mutations.

Keywords: β -Thalassemia; IVSI-110 mutation; Splicing modulation; SR proteins

1. Background

β -Thalassemia is a common genetic disease, affecting many around the world. More than 400 mutations play role in the disease that cause deficit and defect in β -globin in adults. Sever cases of Thalassemia result in manifesting anemia, deformation of bone marrow and splenomegaly that may lead to death in untreated individuals (1). The frequency of β -Thalassemia is very high in Iran and its distribution is not equal in all regions. The highest frequency is more than 10% in areas around Persian Gulf and Caspian Sea (2). More than two million carriers of β -Thalassemia live in Iran (3).

The human β -globin locus extends over 183 kb

with five functional genes (ϵ , $G\gamma$, $A\gamma$, δ and β) on the short arm of chromosome 11 in p15.5. The coding region of β -globin gene in human and other animals is interrupted by two large stretches of non-coding DNA called intervening sequences (IVS) or introns.

Among the mutations in β -globin, some of the most common are: splicing mutations that exist in intron one and two (4). Generally, normal splice sites are not damaged. Thus, a competition between normal and cryptic splice sites is evident. Accordingly, various amounts of normal and aberrantly spliced mRNAs are being produced. Some of the splicing mutations like IVSI-6 produces a large amount of mRNA with normal splicing,

which accordingly causes intermedia β -Thalassemia. However, some of the splicing mutations such as IVS2-654 and IVS I-110 decrease the normal splicing of mRNA molecules to a great extent or sometimes even to zero that leads to Major β -Thalassemia (5).

Splicing is being adjusted through the interaction of many complex splicing factors with various splicing motifs. The main elements of splicing machines are Intronic and Exonic Splicing Enhancers/suppressors (ESE/ESS, ISE/ISS). These elements usually interact with serine/arginine-rich (SR) proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs) (with either positive or negative effect), and central splicing machines such as U1 and U2 small nuclear ribonucleoproteins (snRNPs) (6-8). It was shown that gene expression has been restored through splicing pattern related to hnRNPs up to 80% (9).

Modulation of splicing can be achieved by antisense oligonucleotide, which interacts with sequence-specific splicing motifs (elements) on target RNA. An alternative approach involves activation or suppression of transacting factors such as SR proteins and hnRNPs through drugs. The latter has led to the development of therapeutic approaches using small molecules for modulation of splicing. One such approach involves upregulation of splicing factors to modify splicing, using NaBu, isoBu and VPA, histone deacetylase inhibitors (9, 10).

NaBu has been used to restore normal splicing of cystic fibrosis transmembrane regulator (*CFTR*) transcripts and resulted in functional improvement in the *CFTR* channel in cystic-fibrosis-derived epithelial cell line carrying the 3849 + 10 kb C to T splicing mutation (10). It was found that the mechanism by which NaBu increases the *CFTR* protein, is through increased expression of splicing factors (SC35 and Htra2-b1).

Chang *et al.* (2001). reported the first compound Butyrate drug, which could considerably restore splicing defect in spinal muscular atrophy. NaBu treatment of the transgenic mice model for SMA (Spinal Muscular Atrophy) resulted in an increased expression of survival motor neuron (SMN) protein in the spinal cord and ameliorated neurological symptoms as well as increased acetylation of nuclear histone in neural tissues. The mechanism by which NaBu affects the SMN protein expression of the *SMN2* involves a change in its RNA splicing pattern, which involves the modification of alternative splicing of exon 7 of the *SMN2* under the regulation of SR proteins (9-11). In Cystic Fibrosis, sodium butyrate-a histone deacetylase- increases expression of SR (SC35) and SR-like

(Htra2-B1) proteins, splicing factors that increase exon splicing and restore aberrantly spliced mRNA (12-15).

Butyrates are short chain fatty acids that inhibit histone deacetylase and therefore reactivate many genes (16-19). In hemoglobinopathies, Butyrates are clinically used to treat sickle-cell anemia and Thalassemia through induction of the expression of γ -gene, producing fetal hemoglobin (HbF) and activating the γ -globin (20-24).

In 2011 Hnilicova *et al* using splicing-sensitive microarrays, identified about 700 genes whose splicing was altered after treatment with HDAC inhibitor, NaBu. In cultured HeLa and HeLa-GFP-SRp40 cells, they provided evidences that inhibition of HDAC induced acetylation of histone 4 along with an alternatively spliced element occurs. In addition, they showed that HDAC inhibition reduced co-transcriptional association of the splicing regulator SRp40 with the target fibronectin exon (25).

2. Objectives

Changing the splicing pattern and recognizing the chemical materials that can increase the level of β -globin transcript expression is a fundamental stage in treatment of Thalassemia affected by splicing mutations. IVSI-110 mutation is a common mutation in β -globin gene. In this study, for the first time the effects of Na Bu, isoBu and VPA on the degree of aberrant splicing of IVSI-110 β -Thalassemia pre-mRNA were investigated using primary erythroid cells derived from IVSI-110 β -Thalassemia patients from East Azerbaijan province in Iran. The important novelty of this study is that treatment with these small drugs may increase the levels of β -globin mRNA and blood hemoglobin, reducing the need for frequent blood transfusion.

3. Materials and Methods

In an interventional study, the Human Erythroid progenitor cells were examined to evaluate the effect of the Na Bu and VPA on splicing of IVSI-110 β -Thalassemia pre-mRNA.

3.1. Human Erythroid Cells

Three unrelated β -Thalassemia patients with IVSI-110 mutation, including a female and two males (mean age ≤ 8) were recruited from northwest Iran. Bone marrow (BM) cells of the individuals were collected in Phosphate Buffered Saline (PBS) medium containing 0.5 mL heparin. The cells were precipitated at 1500

rpm for 8 min, treated with 5 mL of Tris Ammonium Chloride buffer (0.01 M Tris-base, 7.4 g.L⁻¹ NH₄Cl, pH 7.2) at 37°C for 5 min. The red blood cells were lysed and washed twice with PBS containing penicillin (100 U.mL⁻¹) and streptomycin (100 µg.mL⁻¹), 2% Fetal Bovine Serum (FBS). The purified BM cells were suspended at 2×10⁶ cells.mL⁻¹ in Dulbecco's Modified Eagle's Medium (DMEM) Medium (GIBCO™, Invitrogen Corporation, Carlsbad, California) containing 30% FBS, L-glutamine (200 mM), penicillin (100 U.mL⁻¹) and streptomycin (100 µg.mL⁻¹), 2 U.mL⁻¹ recombinant human erythropoietin (Stem Cell Technologies, Vancouver, Canada), 150 ng.mL⁻¹ human stem cell factor, 1 µM Dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), Bovine Serum Albumin (BSA) 1% and 2-Mercaptoethanol (1 mM). Erythroid progenitor cells cultured for expansion in 5% CO₂ at 37°C at 2×10⁶ cells per 1 mL/well in 24-well plates; 30% of the medium was replaced daily with fresh medium containing all supplements. The cells were cultured for 4 days.

The cells were washed in PBS containing 2% FBS, containing penicillin (100 U.mL⁻¹) and streptomycin (100 µg.mL⁻¹). The cells were transferred to DMEM differentiation media containing 30% FBS, L-glutamine (200 mM), penicillin (100 U.mL⁻¹) and streptomycin (100 µg.mL⁻¹), 10 U.mL⁻¹ recombinant human erythropoietin (rhEPO), 1% BSA, 1 mM 2-Mercaptoethanol, and 1 mg.mL⁻¹ iron saturated human transferrin. Cells were seeded and maintained at 2×10⁶ cells.mL⁻¹.well⁻¹ of a 24-well plate over 1-2 days of culture.

3.2. NaBu, IsoBu and VPA Administration

Based on unpublished data drawn from transgenic animal study, 0.5 and 1 mM NaBu was added. IsoBu and VPA were added at 0.5 mM and 100 µM, respectively in differentiation media for 24 h and 48 days after plating of cells. Experiments were repeated 3 times.

3.3. Reverse Transcriptase-PCR (RT-PCR) Analysis.

Total RNA was prepared using the Tri-Reagent BD system (Molecular Research Center, Cincinnati, OH) as described by the manufacturer's. First-strand complementary DNA (cDNA) was synthesised using a Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, Calif, USA). Primers 5'-CTGAGGA-GAAGTCTGCCGTTACT-3' (Tm = 60°C) and 5'-CCT-TAGGGTTGCCCATACAG-3' (Tm = 59.3°C) were used to amplify the sequence between exon 1 and 2. The *β-actin* mRNA was used as an internal control and

was amplified with primers *β-actin* (5'-TCCCTGGA-GAAGAGCTACG-3') (Tm = 56.54°C) and *β-actin* (5'-GTAGTTTCGTGGATGCCACA -3') (Tm = 58°C).

To evaluate the effect of the NaBu on splicing, total RNA was extracted from NaBu-treated erythroid cells on days 1, 2 of differentiation. RT-PCR analysis was performed using the IVSI-110 primers to amplify *β-globin*. Meanwhile, *β-actin* was used as a loading control in multiplex RT-PCR reaction.

The PCR (Cinnagen CO, Iran) reaction was performed for 1 cycle at 98°C for 5 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and at 72°C for 45 sec, and a final extension at 72°C for 5 min. The PCR products were analyzed using 3% agarose gel. The size of amplified products were 170 bp and a 189 bp from the *β-globin* gene, which corresponded to the normal and aberrantly spliced mRNA, respectively.

RT-PCR products of *β-actin*, aberrantly and correctly spliced *β-globin* were visualized on a 3% Agarose gel by ethidium bromide staining. Gel scanning with 3D-scanned (BIORAD-imaging system, Life Sciences Inc, USA) and densitometric measurements were carried out with the ONE-Dscan program (Scanalytics, Fairfax, USA). Data analysis was performed using GraphPad Prism software. The relative raw volume amount of each aberrantly and correctly spliced RT-PCR products of *β-globin* were normalized individually to that of RT-PCR product of *β-actin* and presented as mean ± standard deviation. In order to calculate the fold increase in correctly spliced RT-PCR product, the mean normalized raw volume was divided to that of untreated value.

Ethical consideration: This study was approved by the Regional Committee of Ethics of the Tabriz University of Medical Sciences. Written informed consent was obtained from the all participants.

4. Results

4.1. Cumulative Cell Index of IVSI-110 Erythroid Progenitor Cells During the Differentiation Phase Following Treatment with NaBu, IsoBu and VPA

The effects of NaBu, isoBu and VPA on the level of aberrant splicing was investigated in erythroid progenitor cells isolated from IVSI-110 *β*-thalassaemic patients. BM-derived erythroid progenitor cells were subjected to a two-phase liquid erythroid cell culture procedure. Following 4 days of erythroid cell expansion, 2×10⁶ cultured cells were differentiated in the presence of 0.5 and 1 mM of NaBu, 0.5 mM isoBu and 100 µM VA. Daily cell-counts revealed that 0.5 mM

NaBu was well tolerated, while 1 mM reduced cell proliferation and cell viability relative to untreated cells. VA (100 μ M) had slightly lower viability compared to 0.5 mM isoBu, and well-tolerated relative to 1 mM NaBu (Figure 1).

4.2. Correction of IVSI-110 Pre-mRNA Splicing by Pharmacologic Agents in Human Erythroid Progenitor Cells

Using gel electrophoresis, two RT-PCR products corresponding to correctly (170 bp) and aberrantly spliced (189 bp) human IVSI-110 β -globin mRNA were detected (Figure 2). In addition, a single RT-PCR amplification product of the expected size (131 bp) was consistently detected for β -actin in all reactions, indicating that loading and amplification for each sample was equivalent.

Interestingly, RT-PCR of total RNA isolated 24 h after treatment, showed that the level of correctly spliced β -globin mRNA increased in cells treated with NaBu, VPA and isoBu. Concomitant decrease has also been observed in aberrantly spliced mRNA (Figures 2 and 3). In the presence of 0.5 mM NaBu, 100 μ M VPA and 0.5 mM isoBu, the level of normal β -globin RNA increased approximately 1.7 ± 0.5 , 1.5 ± 0.5 and 1.4 ± 0.3 fold relative to untreated cells (Figure 4). These results

showed that, NaBu driven shift in splicing of human β -globin pre-mRNA from aberrant to correct in precursor cells from patient IVSI-110 β -thalassaemic BM was significant.

5. Discussion

Many mutations in the β -Thalassemia diseases are caused by defective splicing of pre-mRNA⁴. A number of these mutations do not alter consensus splice sites or generate missense or nonsense mutations, yet do affect splice site selection (26). IVSI-110 β -thalassaemia splicing mutation, the effects of NaBu, isoBu and VPA on the level of aberrant splicing was investigated in erythroid progenitor cells isolated from IVSI-110 β -thalassaemic patients. In this study, we found that administration of NaBu, VPA and isoBu to erythroid progenitor cells derived from IVSI-110 β -thalassaemic patients resulted in the increased amount of wild-type of β -globin mRNAs 1.7, 1.5 and 1.4 fold respectively relative to normal β -globin mRNAs in untreated erythroid progenitor cells. This considerable increase of expression level in treated cells is reported in the concentration of 0.5 mM, 100 μ M and 0.5 mM from NaBu, VPA and isoBu, respectively. These findings may have important implications regarding the treatment of β -Thalassemia patients.

NaBu and VPA have been shown to increase the expression of some genes by increasing histone acetylation thereby releasing constraints on the DNA template and reactivating a number of genes (27-31). In the context of haemoglobinopathies, utero infusions of Butyrate, delay the developmental switch off γ to β -globin gene expression in sheep fetuses (32). Sodium

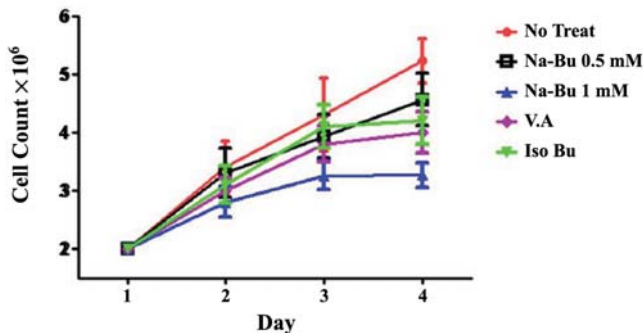


Figure 1. Cumulative index of erythroid progenitor cells during the differentiation phase following treatment with pharmacologic agents

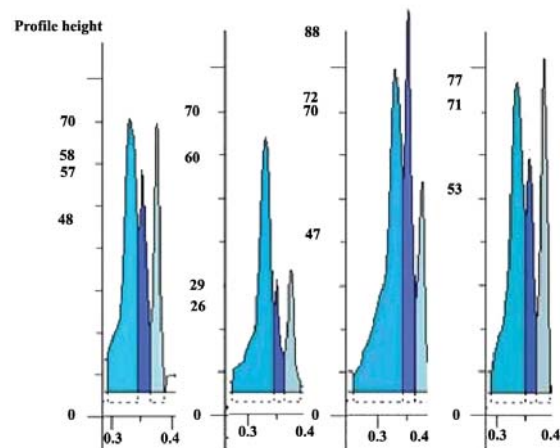
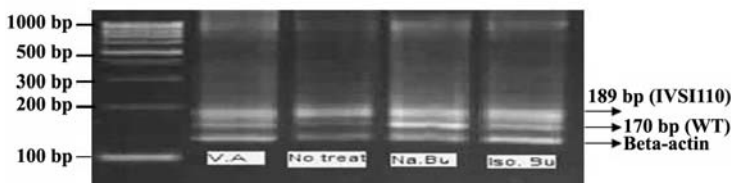


Figure 2. Representative gel electrophoresis (left) and densitometric measurements of RT-PCR analysis (right) of IVSI-110 erythroid progenitor cells treated with NaBu

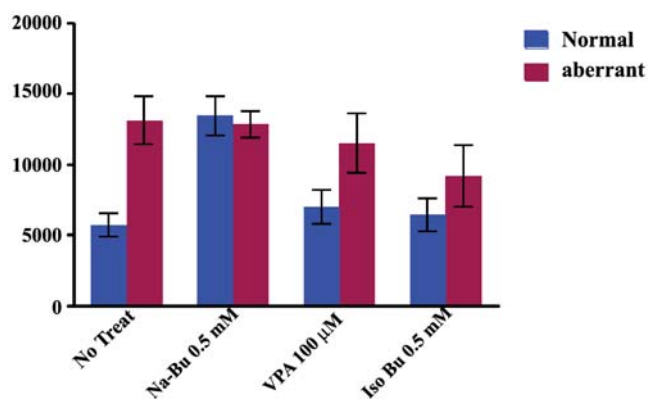


Figure 3. Amount of normal and aberrant +110 β -globin mRNA in cultured BM cells derived from IVSI-110 thalassemic patients following treatment with NaBu, VPA and isoBu

butyrate and similar derivatives increases the expression of fetal-globin genes and are being used clinically in treatment of β -Thalassemia and sickle cell anemia (23, 24, 33). These effects of butyrate may occur through the inhibition of histone deacetylase (30, 31). In a study carried out by Mallca Nissim-Rafina *et al.* (2004), it was revealed that administration of NaBu in cystic fibrosis (CF) increased of Htra2-B1 expression which in turn corrects splicing of *CFTR* gene in CF. In the case of β -Thalassemia patients with IVSI-110 mutation, sodium butyrate may acetylate nucleosomal DNA and release other factors such as Htra2-B proteins, which is in the favor of correct splicing in intron 1 of β -globin gene (34).

Additionally, VPA is a well-known drug confirmed by Food and Drug Administration (FDA) and it has been used for treating epilepsy, bipolar disease, kidney

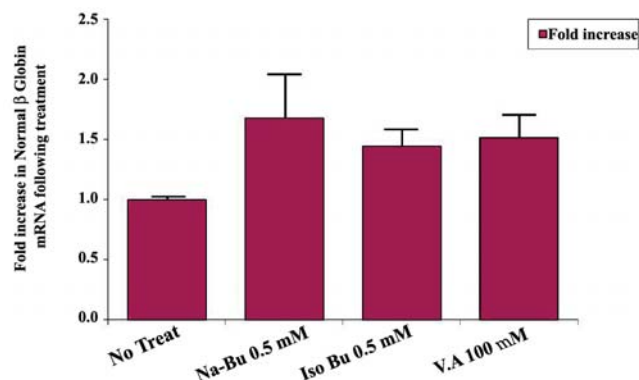


Figure 4. Fold increase of normal β -globin mRNA in cultured BM cells derived from IVSI-110 thalassemic patients following treatment with NaBu, VPA and isoBu

diseases, and migraine in the last three decades(35, 36). Restoring of transcription is among the important effects of the VPA treatment in fibroblast culture of Spinal Muscular Atrophy (SMA) patients (35, 36).

This study suggests that, β -Thalassemia patients with splicing mutations will benefit from NaBu, and VPA drug through two mechanisms: firstly by increasing the HbF and decreasing the α -globin and β -globin imbalance. Secondly, they will increase normal β -globin by restoration of splicing in β -globin gene.

Restoration of β -globin mRNA to 25% of the normal level in Thalassemic patient would have therapeutic potential (37), since heterozygotes with 50% of haemoglobin are frequently asymptomatic.

Furthermore, β -globin mRNA and its protein are very stable and mature erythrocytes have a lifespan of about 120 days. Thus, in principle, treatment with NaBu and VPA may have an extended effect on the *in vivo* levels of β -globin mRNA and blood haemoglobin, reducing the need for frequent administration (29). All the findings from NaBu and VPA treatments *in vitro* and *in vivo* indicate that this drug has a high tolerability in tested systems and can increase restored splicing β -globin transcripts (12). This study showed correction of splicing in human β -globin pre-mRNA driven from IVSI-110 β -Thalassemic patient following treatment with NaBu and VPA. These findings are promising and will help in treatment of Thalassemia through drugs affecting β -globin expression. Meanwhile, this approach may potentially be effective in treating many other forms of thalassemia and inherited monogenic disease caused by splicing mutations.

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