Molecular and cellular pharmacology

Erythroid differentiation ability of butyric acid analogues: Identification of basal chemical structures of new inducers of foetal haemoglobin

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1. Introduction

Several investigations have demonstrated how the clinical status of patients with β-globin disorders can be improved by pharmacologically increased expression of foetal γ-globin genes (Jouini et al., 2012, Musielak, 2011, Huisman, 1979, Stamatoypannopoulos and Nienhuis, 1992 and Gallo et al., 1979). Moreover, foetal haemoglobin levels greater than 9% could reduce early mortality (Platt et al., 1994).

A large number of compounds stimulating foetal haemoglobin production, such as chemotherapeutic agents, 5-azacytidine and hydroxyurea (Bunn, 1997, Ferster et al., 1996, Ballas et al., 2006 and Italia et al., 2013), was considered; however, citotoxicity, potential carcinogenicity and the moderate effects obtained have limited their clinical use (Domenica Cappellini et al., 2000).

The ability to induce foetal haemoglobin was investigated using several molecules, including hematopoietic growth factors and cytokines. For instance, the effects of erythropoietin and interleukin-3 (IL-3) (Breymann et al., 1999 and Reinhardt et al., 2001), as well as the effects of interferon-γ (INF-γ) (Miller et al., 1990) were reported for treatment of the anaemia. In addition, interleukin-4, interleukin-8 and interleukin-18 were found involved with γ-globin gene expression (Kato et al., 2004).

The effects of butyric acid have been investigated since long time in the K562 cell line demonstrating its ability to induce the expression of embryonic globin genes (Cioè et al., 1981). The activity of sodium butyrate and α-amino-n-butyric acid (ABA) to enhance γ-globin synthesis in vitro in erythroid progenitors of patients with sickle cell anaemia and β-thalassaemia, suggested these molecules as relevant for therapy (Perrine et al., 1989). However, there are evidences that the activity of butyric acid and some related compounds can be associated with neurologic toxicity. Therefore, in adult primates, several studies were performed to define the doses able to
induce foetal haemoglobin production at lower concentrations and toxic conditions. In addition, the efficacy and toxicity of butyric acid in the presence of other inducers were evaluated (Blau et al., 1993).

In the pharmaceutical field of investigation, butyric acid represents a prodrug both to develop less cytotoxic compounds (Faller et al., 1993) and increase aqueous solubility for potential clinical applications (Nudelman et al., 2001). For instance, a novel family of butyric acid (acyloxyalkyl analogues) has been synthesised to increase potency ameliorating transport to the cells (Raphaeli et al., 2000).

Among the most studied foetal haemoglobin inducers, histone deacetylase inhibitors (i.e. trichostatin A, arginine butyrate, sodium butyrate, sodium phenylbutyrate) were found to be active in vitro using established cell lines as well as primary erythroid cultures (Glauber et al., 1991, Fibach et al., 1993, Berkovitch-Luria et al., 2012, Muralidhar et al., 2011 and Marianna et al., 2001). The interest in applied biomedicine of butyrates is demonstrated by the several patent applications (few examples of this translational activity are US4822821A, US5645852A, EP0627220A1 and the more recent US8618068).

Several studies report in vivo experiments using both mouse (Pace et al., 1996, Partington et al., 1984 and Perrine et al., 1988), both primates and humans (Constantoulakis et al., 1989a, Lavelle et al., 1993, Constantoulakis et al., 1988, Fucharoen et al., 2013a and Kutlar et al., 2013) models demonstrating the expected clinical effects of this class of foetal haemoglobin inducers. The activity of sodium butyrate and sodium phenylbutyrate and analogues exhibiting similar mechanism of action have been evaluated in clinical trials (Dover et al., 1994, Sher et al., 1995, Collins et al., 1995, Perrine et al., 2011 and Patthamalai et al., 2014) demonstrating induction of foetal haemoglobin in some patients with thalassaemia. A further example in the phase II study made by Domenica Cappellini et al., 2000 made on patients with thalassaemia intermedia treated with oral isobutyramide to evaluate the ability of this butyric acid analogue to stimulate foetal haemoglobin production.

In conclusion, it is well established that the γ-globin gene modulation by butyrate leads to clinically beneficial increasing the endogenous content of foetal haemoglobin. These effects are based on the inhibition of histone deacetylases, causing an accumulation of acetylated histone species in a variety of vertebrate cell lines and hyperacylation of H3 and H4 (Candido et al., 1978), accompanied with deep modification of the chromatin structure and transcriptional events (Cibianelli et al., 2000, Gul et al., 2009, Turner and O’Neill, 1995 and Turner, 1991).

Since the effects of butyrates have been investigated on K562 cells and on primary erythroid progenitor cells (Ciòe et al., 1981, McCaffrey et al., 1997), the in vitro cultures of erythroid precursors represent, in the first step, a model to study haemoglobin production under physiological conditions (Travers et al., 2002; Fibach et al., 2006; Gambari and Fibach, 2007).

The object of our investigation are some analogues presenting chemical structures derived from a compound described in the U.S. Patent number 5,700,640 (23rd December 1997) (Voss and Caron, 1997) and described in a lot of chemical modification in Fig. 1A.

2. Materials and methods

2.1. Compounds employed in the study

The chemical structure of the compound 5049 (Ethyl 4,4,4-trifluoro-butrate) described in the U.S. Patent number 5,700,640 (23rd December 1997) (Voss and Caron, 1997) and the modified analogues were synthesised by Chiesi Farmaceutici SpA (Parma, Italy) and reported in Fig. 1B. These small molecules are similar to butyric acid.

Fig. 1. (A) General formula of the compounds described in U.S. Patent number 5,700,640 (23rd December 1997 by Voss and Caron). (B–D) Chemical structure of the compound 5049 (ethyl 4,4,4-trifluoro-butrate) and the modified analogues 5047, 4174, 4145, 4974, 5043, 5048 (B), of the compounds 5124, 5127, 5128, 5129 and 5130 (C) and of the 4174A, 4174B, 4174C, 4174D and 4174E derivatives (D).
acid, the methyl group replaced with three fluorine atoms producing a fluorinate derivative and the carboxylic esterified with an ethyl group in 5049 compound. The modification of 5047 compound concerns the generation of a double bond between C2 and C3 to produce 2-butenoic acid. The carbonyl skeleton results partially cyclized and forms cyclopropane in 4174 analogue. 4145 compound has a triple bond between C2 and C3 while the carboxylic substituted with sulphonic group in 4974 compound. The 5043 derivative is similar to 4174 compound, but presents a longer carbonyl skeleton with five carbons; the molecule 5048 has five carbons and represents a symmetric molecule containing two acid functions spaced out by a cyclopropane moiety.

In Fig. 1C a second set of compounds and their chemical structures are reported. With respect to butyric acid both 5124 (carboxylic acid) and 5127 (sodium salt) compounds present an additional carbon atom and double bond in C2-C3 and C4-C5 positions. 5128 analogue has double bond in C2-C3 and a methyl group in C3; 5129 is a 5124-like molecule but with an additional carbon atom; the 5130 analogue is a sodium salt of the butyric acid with C3 substituted by a sulphur atom.

We have summarised the last group of studied molecules in Fig. 1D, structurally related to the compound 4174 and purchased from Sigma-Aldrich (St. Louis, MO, USA). All these analogues present a substitution of the carboxylic part: a carbonyl chloride group in 4174A, a carboxamide group in 4174B, a carbonitrile group in 4174C, an isothiocyanato group in 4174D and an ethanone group in 4174E.

All the compounds were prepared in sterile water and the stock solutions were stored at −20 °C in the dark and diluted immediately before use. The biological effects of the compounds at the final concentrations of 0.5, 1, 2.5, 5, 5.6, 7 and 10 mM in cellular cultures were tested. In this study the reference compounds used as erythroid differentiation agents were butyric acid, used at the concentrations of 0.5, 1, 2 mM and thiamycin (MTH) used at 20 mM and purchased from Sigma-Aldrich (St. Louis, MO, USA) (Smith et al., 2000; Fibach et al., 2003).

The erythroid differentiation activity of these small molecules was investigated in human K562 cells by staining with benzidine/ 

| 2.2. Assays of growth inhibition and erythroid differentiation on K562 cell line.

The human leukaemia K562 cells were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% foetal bovine serum (Cebio, Milan, Italy), 50 units/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in an humidified atmosphere of 5% CO2/95% air (v/v) at 37 °C (Lozzio and Lozzio, 1975).

The cells were seeded at 30,000/ml and the treatments were carried out by adding the appropriate drug concentration once at time zero. The cell number/ml was analysed using a model ZF Coulter counter (Coulter Electronics, Hialeah, Fl) after day 2 and day 3 in order to determine possible effects on cell proliferation and the Kc0 value of each compound (the concentration causing 50% growth inhibition) determined.

Erythroid differentiation was assayed by counting benzidine/ 

| 2.3. Cultures of primary erythroid progenitor cells (ErPCs) from healthy donors and β-thalassaemia patients using the two-phase liquid procedure

The employed two-phase liquid culture procedure was previously described (Fibach et al., 2003, 2006; Lampronti et al., 2003).

Following informed consent, peripheral blood samples from healthy donors and β-thalassaemia patients were isolated. The institutional review boards of Ferrara University, Rovigo Hospital and Hadassah Hospital of Jerusalem approved this study.

Mononuclear cells were isolated by lymphocyte-H density gradien
centrifugation (Nycoderm™ polysucrose 400 and sodium diatrizoate Cebio, Milan, Italy), washed three times in 1X PBS solution and cultured for 7 days in α-medium (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% FCS (GIBCO, BRL, Life Technologies, Milano, Italy). 1 μg/ml cyclosporine A (Sigma-Aldrich, St. Louis, Missouri, USA), and 10% conditioned medium from the 5637 bladder carcinoma cell line (Quentmeier et al., 1997). The cultures were incubated at 37 °C, under an atmosphere of 5% CO2 in air with extra humidity. After the first culture phase, the non-adherent cells were harvested, washed and cultured in α-medium with 30% FCS, 1% deionized bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA), 10−8 M β-mercaptoethanol, 2 mM L-glutamine, 10−9 M dexamethasone and 1 U/ml human recombinant erythropoietin (Tebu-bio, Magenta, MI, Italy) and 10 ng/ml stem cell factor (SCF, PeproTech EC Ltd, London, England).

After the second culture phase (7 days long), the compounds to be tested were added and the treatment carried out for further 5 days. The cellular samples were centrifuged, washed and employed for real time RT-PCR reactions.

2.4. RNA isolation from primary erythroid progenitor cell cultures (ErPCs)

The cells were isolated by centrifugation at 1200g for 10 min at 4 °C after washing in 1X PBS and lysed in Tri-reagent™ (Sigma-Aldrich, St. Louis, Missouri, USA). The omogenate was incubated 5 min at room temperature, added with 0.2 ml of chloroform per ml of Tri-reagent™ and vigorously shaken for 15 s, incubated 5 min at room temperature and finally centrifuged at 14,000g for 15 min at 4 °C. The aqueous phase was removed and added with 0.5 ml of isopropanol per ml of Tri-reagent™. After 10 min at room temperature, the samples were centrifuged at 14,000g for 15 min at 4 °C. The RNA pellets were washed with 1 ml of 75% ethanol and centrifuged at 14,000g for 5 min at 4 °C. Finally, the pellets were suspended in RNase-free water to be analysed on 1% agarose gel.

2.5. Real-time quantitative RT-PCR conditions.

For the synthesis of cDNA with random hexamers (TaqMan® Reverse Transcription Reagents, from Life Technologies) 500 μg of total RNA were used. Quantitative real-time PCR assay was carried out using gene-specific double fluorocently labelled probes in a 7700 Sequence Detection System version 1.7 (Life Technologies) (Smith et al., 2000 and Bianchi et al., 2001). The nucleotide sequences used for real-time qPCR analysis of α-, β- and γ-globin mRNAs are here reported: α-globin forward primer, 5'-CAC GCC CAC AAG CTT CG-3', α-globin reverse primer, 5'-AGG GTC ACC AGG AGG CAG T-3', α-globin probe, 5'-FAM-TGG ACC CCG TCA ACT TCA AGC TCC T-TAMRA-3', β-globin forward primer, 5'-CAA GAA AGT CCG TGC T-3', β-globin probe, 5'-FAM-TGG AGG CCT TGG CCA ACT TCA AGC TCC T-TAMRA-3', β-globin reverse primer, 5'-TCA CTC TGG CCA ACT TCA AGC TCC T-TAMRA-3', γ-globin forward primer, 5'-TGG CAA GAA GGT CAC TCT-3', γ-globin reverse primer, 5'-TCA TCT AGG CCG TGA GAG G-3', γ-globin probe, 5'-FAM-TGG GAG ATG CCA TAA AGC TCC TGG TAMRA-3' (Fibach et al., 2003). The fluorescent reporter and the quencher were: 6-carboxyfluorescein (FAM) and 6-carboxy-NN,N',N-trimethylrhodamine (TAMRA), respectively. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, fluorescently-labelled with VIC™ (Life Technologies, Applied Biosystems, Foster City, CA, USA) were used as reference genes.
2.6. High-performance liquid chromatography of human erythroid precursor lysates

The cells were harvested, washed once with 1X PBS, and the pellets were lysed in water by freeze and thaw cycles and recovered by centrifugation at 14,000g for 30 min and –4 °C. The supernatants (haemoglobin proteins) were collected and injected on a Syncropak CCM 103/25 (250 mm × 4.6 mm) column (Eichrom Technologies, Inc.) and separated by cation-exchange HPLC using a Beckman Coulter instrument System Gold 126 Solvent Module-166 Detector. Samples were eluted by solvent gradient (aqueous sodium acetate-BisTris-KCN buffers) and detection was performed at 415 nm. The standard controls were the purified adult haemoglobin (SIGMA, St. Louis, MO) and foetal haemoglobin (Alpha Wassermann, Milano, Italy) (Salvatori et al., 2009).

3. Results

3.1. Effects on proliferation and erythroid differentiation of human K562 cells

The growth of K562 cells was analysed after treatment with the investigated compounds for 1, 2 and 5 days. Representative results obtained after 2 days of culture are presented in Fig. 2, where in panel A the growth kinetics of untreated K562 cells untreated and K562 cells treated with the compounds 5049, 5047, 4174, 4145, 4974, 5043 and 5048 are reported. Similarly, in Fig. 2B are indicated the representative cell growth kinetics for the compounds 5124, 5127, 5129, 5128, 5130 and butyric acid. The results obtained firmly demonstrate that most of the compounds cause a dose-dependent inhibition of cell proliferation, with the exception of analogues 5047, 4174, 4145, 4974 (Fig. 2A), and 5128 (Fig. 2B). The extrapolated IC50 values relative to each analogue are reported in Table 1.

The same molecules were tested for their ability to induce erythroid differentiation of the K562 cell line measuring the intracellular haemoglobin (Hb)-content by the benzidine/H2O2 procedure (Bottini et al., 1963 and Preisler and Giladi, 1975). The compounds were assayed at the concentrations of 0.5, 1, 2.5, 5, 5.6, 7 and 10 mM and the benzidine-positive cells were evaluated at 5, 6 and 7 days from the onset of the treatment. Representative results are shown in Table 2. The data obtained at day 7 suggest that the best erythroid differentiation agent (B+ cells=55%) is the compound 4174, containing a cyclopropane ring obtained by cyclization of butyric acid. This erythroid induction is not associated with cytotoxic effects, since 4174 has no anti-proliferative activity (see Fig. 2A). In addition to compound 4174, also compound 5124 should be considered as a good inducer (B+ cells=47%). Interestingly, this molecule presents a C4 chain and two double bonds in C2–C3 and C4–C5 positions, and it is structurally related to valeric acid, known to be a good erythroid inducer in different model systems (Voss and Caron, 1997 and Safaya et al., 1994). Unlike compound 4174, 5124 exhibits a lower IC50 value, indicating inhibitory effects on cell growth (Table 1). In any case, the analogues 4174 and 5124 exhibit erythroid induction properties even better than butyric acid (Table 2).

As far as the other studied analogues, compound 5130 displays an induction activity similar to butyric acid and represents a sodium salt analogue of the methyl thioacetic acid, an inducer reported by Voss and Caron (1997). On the contrary, compounds 5049, 4145 and 4974 despite being structurally related to the general formula described in the patent are less effective than butyric acid. Among the assayed molecules, 5043 and 5048, containing a cyclopropane ring as 4174, exhibit lower activity than butyric acid. This suggests that the 4174 induction activity is reduced by carbonyl skeleton elongation as in compound 5043 and by addition of a carboxyl group as in 5048.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>%B+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5049</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>5047</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>4174</td>
<td>5.6</td>
<td>55</td>
</tr>
<tr>
<td>4145</td>
<td>5.6</td>
<td>18</td>
</tr>
<tr>
<td>4974</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>5043</td>
<td>2.5–7</td>
<td></td>
</tr>
<tr>
<td>5048</td>
<td>5.6</td>
<td>15</td>
</tr>
<tr>
<td>5124</td>
<td>5</td>
<td>47</td>
</tr>
<tr>
<td>5127</td>
<td>0.5–10</td>
<td>1</td>
</tr>
<tr>
<td>5128</td>
<td>5.6</td>
<td>8</td>
</tr>
<tr>
<td>5129</td>
<td>0.5–10</td>
<td>1</td>
</tr>
<tr>
<td>5130</td>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>2.5</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 1
IC50 values of the assayed compounds in K562 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5049</td>
<td>7.90 ± 0.34</td>
</tr>
<tr>
<td>5047</td>
<td>33.10 ± 2.15</td>
</tr>
<tr>
<td>4174</td>
<td>31.80 ± 1.72</td>
</tr>
<tr>
<td>4145</td>
<td>27.90 ± 1.66</td>
</tr>
<tr>
<td>4974</td>
<td>25.50 ± 1.91</td>
</tr>
<tr>
<td>5043</td>
<td>5.70 ± 0.55</td>
</tr>
<tr>
<td>5048</td>
<td>6.10 ± 0.73</td>
</tr>
<tr>
<td>5124</td>
<td>6.46 ± 0.28</td>
</tr>
<tr>
<td>5127</td>
<td>3.73 ± 0.22</td>
</tr>
<tr>
<td>5128</td>
<td>19.45 ± 0.74</td>
</tr>
<tr>
<td>5129</td>
<td>4.17 ± 0.14</td>
</tr>
<tr>
<td>5130</td>
<td>7.66 ± 0.79</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>3.22 ± 0.11</td>
</tr>
</tbody>
</table>

Table 2
Benzidine/H2O2 activated (%B+ ) cells of the assayed compounds in K562 cells.

Fig. 2. Decrease of cell proliferation of K562 cell cultures treated with the compounds 5049, 5047, 4174, 4145, 4974, 5043 and 5048 (see panel A) and with the compounds 5124, 5127, 5129, 5128 and 5130 at the indicated concentrations in comparison with butyric acid (see panel B) (average ± S.D. from three independent experiments).
Finally, compounds 5047, 5127, 5128, 5129 have no effects on erythroid differentiation of K562 cells (B⁺ cells < 10%) (Table 2). The 5047 and 5128 analogues contain a double bond C2–C3 (Fig. 1B and C) with respect to the chemical structure of butyric acid and present a reduced anti-proliferative and erythroid differentiating activity; in these analogues, the addition of a methyl group could increase the growth inhibition ability (Tables 1 and 2). In addition, when the carbonyl skeleton is one atom longer than in butyric acid and contains two double bonds in C2–C3 and C4–C5 positions (such as in the 5124 analogue), the erythroid differentiation is significantly increased (B⁺ cells = 47%), but this effect is completely abolished if the carboxylic acid is converted into carboxylate sodium salt (as it occurs in compound 5127). Similarly, we have the same negative effect by carbonyl skeleton elongation as obtained using 5129 (Table 2).

In conclusion, the experimental approach, despite it is based on a limited structure–activity relationship analysis using the cell proliferation and erythroid differentiation assays, is a useful tool (a) to study chemical residues affecting erythroid differentiation induction and (b) to identify, among a variety of molecules, the best erythroid inducer. Among the studied compounds, 4174 should be considered the best one, exhibiting erythroid differentiation activity without showing any anti-proliferative effects, unlike 5124, that in capable to induce erythroid differentiation, but at the same time exhibits strong antiproliferative effects.

3.2. Effects of 4174 and derivatives on human erythroid precursor cells

Before performing extensive analysis on erythroid precursor cells from β-thalassaemia patients, five derivatives of compound 4174 were synthesised by Chiesi Farmaceutici SpA (Parma, Italy), as reported in Fig. 1D, in order to find an even better erythroid inducer. The analogues 4174A, 4174B, 4174C, 4174D and 4174E present a cyclopropane and the carboxylic group substitution with no acid portion. All the compounds have similar chemical structure to 4174 and they were synthesised to find an even better erythroid inducer.

We next studied the effects of 4174 and derivatives on γ-globin mRNA accumulation in erythroid precursor cells (ErPCs) from normal donors cultured according to the two-phase liquid culture protocol (Fibach et al., 2006, Mischiati et al., 2004 and Lampronti et al., 2003). In this procedure, early erythroid committed progenitors derived from the peripheral blood of healthy donors proliferate and differentiate during phase 1 (in the absence of erythropoietin) into late progenitors (erythroid colony-forming units, CFU-Es). In phase 2, in the presence of erythropoietin, the latter cells continue to proliferate and mature into haemoglobin-containing orthochromatic normoblasts.

The compounds 4174 (7 mM), 4174 A (10 mM), 4174B (5.6 mM), 4174 C (7 mM), 4174D (2 mM), 4174E (2 mM) and butyric acid (1.5 mM), a known stimulator of foetal haemoglobin production in vitro and in vivo (Domenica Cappellini et al., 2000; Berkovitch-Luria et al., 2012; Perrine et al., 2011; Gabbianelli et al., 2000 and Johnson et al., 2005), were added on day 5 of phase two (when cells started to synthesise haemoglobin), and the cells were harvested on day 12. After treatment, the effects on α-globin, β-globin and γ-globin mRNAs were analysed by quantitative RT-PCR. Fig. 3A shows the increase in the content of α-globin, β-globin and γ-globin mRNAs in treated cultures (using GAPDH as reference gene). The data obtained demonstrated that, with the exception of 4174B and 4174D, all the compounds are active in inducing γ-globin mRNA production. The most powerful inducers were found to be, together with 4174, 4164A and 4174C. However, as shown in Fig. 3A and further detailed in Fig. 3B, 4174A and 4174C induce also an important increase of α-globin mRNA. Since this should be avoided in the treatment of β-thalassaemia, the further characterisation was conducted on 4174.

In order to relate γ-globin gene expression with foetal haemoglobin production, HPLC analysis was performed (Fig. 4). The foetal haemoglobin content was determined in healthy ErPCs treated with either 4174 compound (15, 20, 25 mM) or butyric acid (0.5 and 0.8 mM) from day 5 to day 12 of phase 2. The results in Fig. 4 represent the percentage of foetal haemoglobin with respect to total haemoglobins. The results demonstrate that foetal haemoglobin increases from 2.3 ± 0.1% in control cells to 7.6% at 0.5 mM in butyric acid-treated cells and to 13.1% at 20 mM in 4174-treated cells. The fold increase of foetal haemoglobin in 4174-treated cells

![Fig. 3](image-url) A. Quantitative RT-PCR analysis of α-globin (white), β-globin (light grey) and γ-globin (dark grey) mRNAs in treated and untreated precursor cultures from healthy subjects. Data represent the fold increase of globin transcripts using GAPDH as reference gene and taking untreated ErPCs as reference control (1.0). B. Increase of the ratio of β-globin/α-globin (light grey) and γ-globin/α-globin (dark grey) mRNAs induced by the reported molecules. Butyric acid was taken as positive control.

![Fig. 4](image-url) Foetal haemoglobin induction obtained following treatment of ErPCs from healthy subjects with compound 4174 (black) and butyric acid (taken as positive control) (light grey) at the indicated concentrations and without treatment (white).
4174 was used at 7 mM on ErPCs from seven β-thalassaemia patients and treated with compound 4174. Data represent the fold increase of globin transcripts using GAPDH as internal gene control and taking untreated ErPCs as reference control (average ± S.D. from four independent PCR reactions using precursor cultures from the same patients).

Correlates with the increase of γ-globin mRNA content and is much more evident than in the case of butyric acid induction.

3.3. Compound 4174-mediated induction of γ-globin gene expression in erythroid precursor cells from β-thalassaemia patients

The effects of compound 4174 on γ-globin mRNA accumulation was assayed on erythroid precursor cells from β-thalassaemia patients using phase 1 and 2 cultures and described previously (Fibach et al., 2003, 2006 and Lampronti et al., 2003). Compound 4174 was used at 7 mM on ErPCs from seven β-thalassaemia patients and the effects on γ-globin and α-globin mRNAs were analysed by quantitative RT-PCR and reported in Fig. 5. The results show that with the exception of th2 and th6, the compound 4174 produced a sharp induction of γ-globin gene expression. Interestingly, in all the responsive ErPC cultures (th1, th3, th4, th5 and th7) this compound did not produce any inducing effect on α-globin gene expression. The finding that ErPCs from some patients (th2 and th6) exhibit low response suggest that this approach can be employed as a useful model to assay for pharmacological responsiveness in vitro.

4. Discussion

Butyric acid analogues are, together with hydroxyurea, the most used foetal haemoglobin inducers for thalassaemia and sickle cell anaemia therapy (Gambari and Fibach, 2007). The clinical and translational interest on these molecules is demonstrated by several granted patents (a limited examples are US4822821A, US5645852A, EP0627220A1) (Perrine, 1989; Newmark, 1989; Hayhurst et al., 1994). A key example is the recently developed sodium 2,2 dimethylbutyrate (HQK-1001), object of patenting (US8618068) (Perrine et al., 2013). Among the investigated compounds, 4174 resulted the best γ-globin and foetal haemoglobin inducer using this multistep approach among. In particular, the ability of this molecule to induce foetal haemoglobin has never been investigated and demonstrated. The only effects reported in the literature so far are as hypoglycaemia agent inhibiting the metabolism of various substrates via the mitochondrial monocarboxylate transporter and the fatty acid oxidation (Buxton et al., 1983). Moreover, the cyclopropane carboxylate has effects on gluconeogenesis and pyruvate decarboxylation as well, suggesting that, through undefined mediators, it inhibits pyruvate decarboxylation and gluconeogenesis acting on the pyruvate cycle when pyruvate supports gluconeogenesis (Steinhelper and Olson, 1985).

When ErPC from β-thalassaemia patients were used, compound 4174 was found to efficiently induce the expression of γ-globin genes, despite with some degree of variability (ErPCs from two β-thalassaemia patients did not respond to the treatment) (Fig. 5). This finding is of interest, since it is well known that γ-globin gene increase can be proposed in the therapy of thalassemic patients (Ballas et al., 2006, Italia et al., 2013, Fathallah and Atweh, 2006, Fathallah et al., 2009, Fathallah et al., 2013).
Steinberg, 2003 and Bhatia et al., 2009). In this context, our method finds implications in tailored therapies, particularly in the early steps before setting clinical trials (Fuchareon et al., 2013a, Kutlar et al., 2012 and Patrinos and Grosvold, 2008).

In conclusion, the application of the methods employed allowed us to find possible correlations between chemical structure modifications and foetal haemoglobin inducing activity. The structural analysis of the most effective molecules suggests a new basal chemical structure in which it is possible to introduce changes for the developing of compounds that are more effective. This approach represents a reproducing system of multistep cultures for the screening of foetal haemoglobin inducing agents for the development of novel approaches in the therapy of haematological disorders, including β-thalassaeia and sickle cell anemia. Finally, the individual response to these treatments may be a starting point for pharmacogenomic studies.

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