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Full Length Article

Epigenetic and Transcriptional Modulator Potential of Epigallocatechin-3-gallate and Genistein on Fetal Hemoglobin Reactivators Genes

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ABSTRACT

Background: β-hemoglobinopathies are one of the most common recessive genetic diseases worldwide, with limited treatments available, particularly in developed countries where the prevalence is higher. Pharmacological reactivation of Fetal Hemoglobin (HbF) is a promising therapeutic strategy. However, approximately 25% of the patients do not respond to Hydroxyurea (HU), the first and most commonly used HbF inducing agent approved by the FDA.

Objective: Here, we performed an *in vitro* assessment of transcriptional effects induced by natural bioactive compounds, namely Epigallocatechin-3-gallate (EGCG) and genistein (GN) in globin genes (*HBA1, HBB, HBG1* and *HBG2)* in HbF regulators/silencer genes (*KLF1, BCL11A, MYB* and *BGLT3)* and in epigenetic regulator genes (*DNMT1, DNMT3A, DNMT3B, HDAC1, HDAC2, HDAC3* and *HDAC8*). Moreover, we evaluated EGCG's *in vivo* effects in hematological parameters of healthy volunteers.

Methods: K562 cells were exposed for 72 and 96 h to GN and EGCG at 100, 250 and 500 ng/mL. Cell proliferation and viability were measured, and transcriptional levels were evaluated by qRT-PCR. For *in vivo* assay, complete blood count was determined by flow cytometry and HbF level was determined through HPLC in 30 healthy individuals before and after 225 mg EGCG ingestion per day during a 90-day period.

Results: Both compounds impact cellular metabolism and proliferation with no cytotoxic effects. Divergent GN and EGCG effects in globin and *BGLT3* expression levels suggest the involvement of divergent signaling pathways. As for the epigenetic potential, EGCG particularly affects *HDAC2* and *HDAC8* transcription, whereas GN significantly affects expression patterns of methylation and acetylation modulators. HU appears to have time divergent effects, with greater impact in methylation at 72 h (overregulates *DNTM3A*) while affecting acetylation mostly at 96 h (downregulates *HDAC1* and *HDAC8*). Additionally, *in vivo,* EGCG demonstrated a modulator effect in hematopoiesis and HbF induction.

Conclusion: Our results advocate EGCG and GN with HbF pharmacological reactivation potential and sustain further research as new alternative approaches for β-hemoglobinopathies therapies.

1. Introduction

Hemoglobinopathies are one of the most common autosomal recessive diseases worldwide, affecting about 300,000 newborns every year, mostly with SCD (83%) and thalassemias (17%) (Goonasekera et al., 2018). Considering that approximately 24% of the world's population are carriers of hemoglobinopathy-causing genomic variants (Goonasekera et al., 2018), these diseases are expected to rise by 2050 (Biswas, 2013).

β-hemoglobin disorders are characterized by structural abnormalities in the globin proteins resultant from the deficient or altered synthesis of the β-globin chain of human hemoglobin. β-thalassemia and Sickle Cell Disease (SCD) are the most predominant β-hemoglobin disorders, particularly in the Mediterranean, Africa and Southeast Asia

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(Sankaran, 2011). SCD is found in 83% of patients diagnosed with hemoglobinopathy (Goonasekera et al., 2018). The substitution of valine for glutamic acid in the sixth position of the β globin chain results in the synthesis of abnormal hemoglobin (HbS) molecules that under hypoxic conditions, polymerize inside the cells, forming rigid, sickled cells. The homozygous state of SCD (SCD-SS or SCA) is associated with severe health complications and a reduced life expectancy (Sankaran and Orkin, 2013).

Currently, there are several available treatments for these disorders, including bone marrow transplantation, blood transfusions, and gene or cellular therapies, which however are limited by drawbacks such as being applied only for a minority of patients (Lawson et al., 2003), complications (Marsella, 2014) and being still in experimental stages and have faced numerous challenges (Demirci et al., 2018; Field and Nathan, 2014).

Additionally, the widespread use of these treatments has major limitations, particularly in developing countries where affected patients are frequently unable to sustain the high costs of clinical management.

For the past decades, pharmacological reactivation of Fetal Hemoglobin (HbF) has been seen as a promising therapeutic strategy for patients with these conditions based on the observation that hereditary persistence of HbF minimizes the severity associated symptoms and that higher levels of HbF in F cells (HbF containing red blood cells) are correlated with increased cell survival in patients with SCD (Shaukat et al., 2018). In humans, HbF predominates during mid to late gestational stages and persists at a low level postpartum in mature erythroid cells after adult hemoglobin (HbA) prevails (Sankaran and Orkin, 2013). However, HbF levels vary significantly (1–25%) in individuals with SCD-SS and act as a quantitative genetic trait.

The ideal target for the treatment of β-hemoglobin disorders would be one that mimics and boosts the effect of the genetic variants that regulate HbF levels, such as B-cell lymphoma-leukemia A (*BCL11A*) transcription factor silencer (Sankaran, 2011; Sedgewick et al., 2008) and the HBS1LMYB polymorphism located at the promoter region of the *HBG2* gene (Pereira et al., 2015) without affecting other biological pathways. Furthermore, recently a new regulatory element has been described, namely the noncoding *BGLT3* gene, which regulates chromatin architecture and LCR-γ-globin contact, a crucial regulatory region seen as a prospective target for therapeutic genome editing (Huang et al., 2017).

Hydroxyurea (HU), a potent ribonucleotide reductase inhibitor initially developed as an antineoplastic agent, is currently the most commonly used treatment for SCD patients (Field and Nathan, 2014). HU induces γ-globin gene and reduces β-globin gene expression via p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway (Chou et al., 2015). Other molecules, including natural bioactive compounds, that act in the same pathway with low cytotoxicity could be interesting targets for new drugs development. Genistein (GN), a naturally occurring flavonoid found in soybean and soy derivates, regulates mitogen-activated protein kinase (MAPK) pathways, such as the p38 MAPK and ERK1/ERK2 and endorses a strong immune modulatory effect on the macrophages (Cui et al., 2014). GN constitutes an important component in the majority of people's daily diet and is one of the most studied isoflavones associated with a variety of biological activities, including as a phytoestrogen, an antioxidant, and as an inhibitor of a broad range of tyrosine kinases (Ganai and Farooqi, 2015).

Another molecule is epigallocatechin-3-gallate (EGCG), the major polyphenol component of green tea with potent antioxidant and anti-inflammatory properties (Chu et al., 2017), of which health benefits have been associated with its histone acetyltransferases (HATs) and histone deacetylases (HDACs) effects (Vahid et al., 2015) and DNA methylation (Negri et al., 2018). Additionally, EGCG also triggers p38 MAPK signaling pathway (Shu et al., 2018). Moreover, EGCG was reported to effectively inhibit *in vitro* dehydration of sickle red blood cells (Ohnishi et al., 2001) and decrease oxidative stress in iron-treated erythrocytes (Thephinlap et al., 2007).

Both EGCG and GN have also been identified as epigenetic modulators, reactivating the expression of tumor-suppressing genes in different types of cancer mainly through enzymatic inhibition of DNA methyltransferases (DNMT) and HDAC (Fang et al., 2007; Kato et al., 2008; Khan et al., 2015; Majid et al., 2010; Nandakumar et al., 2011; Xie et al., 2014).

The aim of this study was to perform an *in vitro* assessment of potential transcriptional effects induced by EGCG and GN exposure in globin genes (*HBA1, HBB, HBG1* and *HBG2*), HbF regulators/silencer genes (*KLF1, BCL11A, MYB* and *BGLT3*) and epigenetic regulator genes (*DNMT1, DNMT3A, DNMT3B, HDAC1, HDAC2, HDAC3 and HDAC8*), in the established K562 cell line previously utilized in several studies of fetal hemoglobin induction (Li et al., 2018; Qian et al., 2013; Witt et al., 2003). Moreover, we also evaluate the *in vivo* effect of EGCG intake during 90 days in hematological parameters, including HbF after ingestion of 225 mg EGCG per day.

2. Materials and Methods

2.1. In vitro assay

2.1.1. Cell cultures and reagents

K562 cell line (ECACC No. 89121407) derived from human chronic myelogenous leukemia (CML) in blast crisis was purchased from the European Collection of Cell Cultures (ECACC, UK). K562 cell line was chosen due to the fact that it was previously utilized in several studies of fetal hemoglobin induction (Li et al., 2018; Qian et al., 2013; Witt et al., 2003), including CRISPR-Cas9 genome-editing with deletion of *BCL11A* associated with a strong induction of γ-hemoglobin expression (Khosravi et al., 2019). Additionally, similar results regarding γ-globin induction associated with *BCL11A* down-regulation in the K562 cell line and human erythrocytes from normal donors and β-TM donors (Li et al., 2018) validated beyond doubt the utility of this cellular model. Cells were cultivated in 75 cm^2 flasks with RPMI 1640 (Sigma-Aldrich) media containing GlutaMAX-l, 25 mM HEPES (Invitrogen, USA) prewarmed at 37°**C** and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM *L*-glutamine (G6784, Sigma-Aldrich). Cultures were maintained in a 5% (v/v) CO_2 humidified atmosphere at 37°**C**. Cells were stabilized between the density of 1×10^5 and 1×10^6 cells/mL, performing every three days subcultures and examining microscopically (phase contrast) regarding their confluence, number, morphology, and viability. Subculture cells were allowed to stabilize for 24 h in a standard growth medium before treatments. For treatments and experiments, K562 cells were used between passages 1 and 14.

2.1.2. Drugs and treatments

Epigallocatechin-3-gallate (EGCG) (CAS number 989-51-5; Sigma-Aldrich, USA) and genistein (GN) (CAS No. 446-72-0; Sigma-Aldrich, USA) were purchased and stored at 5 mg/mL stock solutions. EGCG was dissolved in sterile water and DMSO was used as GN vehicle. Working solutions performed with RPMI 1640 with ECGC and GN final concentrations of 100, 250 and 500 ng/mL.

For experiments, K562 cells at a density of 1×10^4 cells/mL were exposed to EGCG and GN and seeded in 6-well plates (3 mL/well) for transcriptional effects assessment and in 96-well plates (100 μL/well) for evaluation of the potential for cytotoxic effects.

In this study Hydroxyurea (HYDREA®, USP), a known HbF inducer, was used as a positive control at a final concentration of 25 μg/mL. As negative control cells were grown in standard culture medium, and in culture medium supplemented with 500 ng/mL DMSO which corresponds to the final concentration of the vehicle solution in GN exposed cells.

Exposed cells and controls were incubated at a humidified atmosphere of 5% CO_2 at 37°**C** and harvested for post-treatment assays and analysis at 72 and 96 h. At least three biological replicates and three

Table 1
Primer sequences, accession numbers and product lengths for qRT-PCR analysis.

Genes	Accession Number*	Forward Primer (5′→ 3′)	Reverse Primer (3′→ 5′)	Product Length (bp)
GAPDH	NM_002046.7	GAGTCAACGGATTTGGTCGTA	GCAGAGATGATGACCCTTTTG	245
HBA1	NM_000558.5	TCCCCACCACCAAGACCTAC	CCTTAACCTGGGCAGAGCC	63
HBB	NM_000518.5	GCACGTGGATCCTGAGAACT	GCCACCACTTTCTGATAGGC	117
HBG1	NM_000559.3	TGGATGATCTCAAGGGCAC	TTGCAGAATAAAGCCTATCCTTGA	258
HBG2	NM_000184.3	GATGCCATAAAGCACCTGGATG	TTGCAGAATAAAGCCTATCCTTGA	274
KLF1	NM_006563.5	GGTGTGATAGCCGAGAC	GCGTATGGCTTCTCCC	164
BCL11A	NM_022893.4	ATTCGGCGTAGTACCC	CAACGGCTTCTTGGAG	191
MYB	NM_001130172.2	CCCAAGTCTGGAAAGCGTCA	TTCGATTCGGGAGATAATTGGC	130
BGLT3	NR_121648.1	ACAAACCAGCATCCTGAACC	GTCTCATGTGCTGCACGTCT	148
DNMT1	NM_001379.4	CCTCCAAAAACCCAGCCAAC	TCCAGGACCCTGGGGATTTC	101
DNMT3A	NM_022552.5	CCAACATCGAATCCATGAAA	CTTGCGCTTGCTGATGTAGT	140
DNMT3B	NM_175850.3	CGAATTTTACCACCTGCTGAATT	AGAACGGCCGGTCATCAC	59
HDAC1	NM_004964.3	GGAAATCTATCGCCCTCACA	AACAGGCCATCGAATACTGG	168
HDAC2	NM_001527.4	CTGTTAATTGGGCTGGAGGA	AATTCAAGGATGGCAAGCAC	94
HDAC3	NM_001355040.2	GGACCAGATCCTCCAGACAA	CAGCCTCATCAGTCCTGTCA	116
HDAC8	NM_001166418.2	GGTGACGTGTCTGATGTTGG	AGCTCCCAGCTGTAAGACCA	165

*NCBI Reference Sequence (National Center for Biotechnology Information).

technical replicates were performed for each compound and concentration.

2.1.3. Cell proliferation and viability assays

Cellular proliferation was assessed through trypan blue exclusion assay (Sigma, St. Louis, USA). Cellular viability was evaluated through the CellTiter-Blue® Cell Viability Assay (Promega) according to the manufacturer's instructions. Standard culture media and reagent were used as a "white's medium" (control). Viability was determined by measuring absorbance using a microplate reader spectrophotometer (Optic Ivyman; System 2100C, Spain). Experiments were performed in the three replicates *per* treatment.

2.1.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted using the SV Total RNA Isolation System (Promega, USA), according to the manufacturer's instructions.

All RNA samples concentration was determined by a fluorescence-based assay, the Qubit™ RNA HS Assay Kit in Qubit™ 3.0 Fluorometer (Invitrogen, USA), and 2.0 μg of total RNA from each sample were reverse transcribed into cDNA using the Applied Biosystems™ TaqMan™ Reverse Transcription Reagents (Invitrogen™, *Thermo Fisher Scientific Inc.*, USA), with random hexamers as primers, in a scaled-down (20 μL) RT reaction, according to the manufacturer's instructions. The RT reactions used the following conditions: 25°**C** for 10 min, 37°**C** for 30 min, 95°**C** for 5 min, hold at 4°**C**.

Quantitative real-time PCR (qRT-PCR) was performed on a CFX Connect™ Real-Time PCR Detection System (Bio-rad, USA) to quantify gene expression using *GAPDH* as a reference gene. Each reaction was performed in triplicate using the iTaq Universal SYBR Green Supermix (Bio-rad, USA) in a final volume of 20 µL. Control PCRs were also performed for all primer combinations without template. The specific primers used are listed in Table 1 and the cycling conditions were as follows: 95°**C** for 2 min; and 45 cycles of 95°**C** for 30 s, 55°**C** for 30 s, 72°**C** for 1 min with fluorescent reading. Relative quantification was undertaken by normalizing threshold cycles (Ct) of the target genes with the mean Ct of *GAPDH* transcript levels were analyzed by calculating ΔΔCt (ΔΔCt=ΔCt treatment−mean ΔCt control). The obtained ΔΔCt values were thereafter $\log_2$-transformed for graphical proposes.

2.2. In vivo assay

2.2.1. Study population and design

This was an interventional, uncontrolled, prospective, longitudinal and individual analysis study, which included 30 healthy individuals (with no previously diagnosed pathologies). Inclusion criteria considered were adult volunteers (ages superior to 18 years old and less than 65 years old) with no acknowledged previously diagnosed pathology of any type. Exclusion criteria applied were actual viral infections, regular consumption of tea and forgetting to take capsules on consecutive days during the study. Data were analyzed under blind conditions.

2.2.2. Supplementation and questionnaires

Commercial capsules of green tea extract with 225 mg EGCG/capsule (My Protein®) were provided for all the participants in the study with the instructions to take 1 capsule daily. EGCG dosage was selected considering that, 400 mg and 800 mg of EGCG intake, which are considered safe doses previously used in human clinical trials (Identifier: NCT00942422, n.d.), result in peak serum concentrations in the range of 100–400 ng/mL (Chow et al., 2003). Thus, the selected dosage is considered safe. The participants filled up 2 questionnaires, the first in the moment of the first blood collection (T0; June), with questions regarding demographic data such as age, gender, weight (kg), height (cm) and smoking habits. In the second questionnaire, after 90 days (T90; September) of the interventional study, the participants were asked to report adverse effects through the intervention, namely alterations in the nervous system (headaches, migraines, mood swings); gastrointestinal system (heartburn, reflux, diarrhea, cramps, weight loss/gain); cardiac and respiratory effects and information regarding potential missing capsules intake.

2.2.3. Collection of biological samples

Peripheral blood of the volunteers was collected in a tube with anticoagulant ethylenediaminetetraacetic acid (EDTA) VACUETTE® for analysis of hematological parameters using standard blood collection procedures at time 0 (T0). Over the following 90 days, participants ingested commercial capsules of green tea extract (225 mg EGCG/capsule; Myprotein) daily. After 90 days of exposure, another blood collection was performed (T90). All samples were refrigerated at 4°**C** until processed in the hematology laboratory.

2.2.4. Hematologic parameters

Complete blood count was evaluated by flow cytometry in Advia 120 (Siemens, Germany) equipment and HbF determined through high-performance liquid chromatography (HPLC) in Arkray HA-8180 (Menarini, Italy) equipment, in General Lab Portugal.

2.3. Statistical analysis

For transcriptional assays, statistical analysis was performed using IBM® SPSS® Statistics 25 software (Armonk, NY, USA). The significant differences between the two treatments, cells grown in standard medium

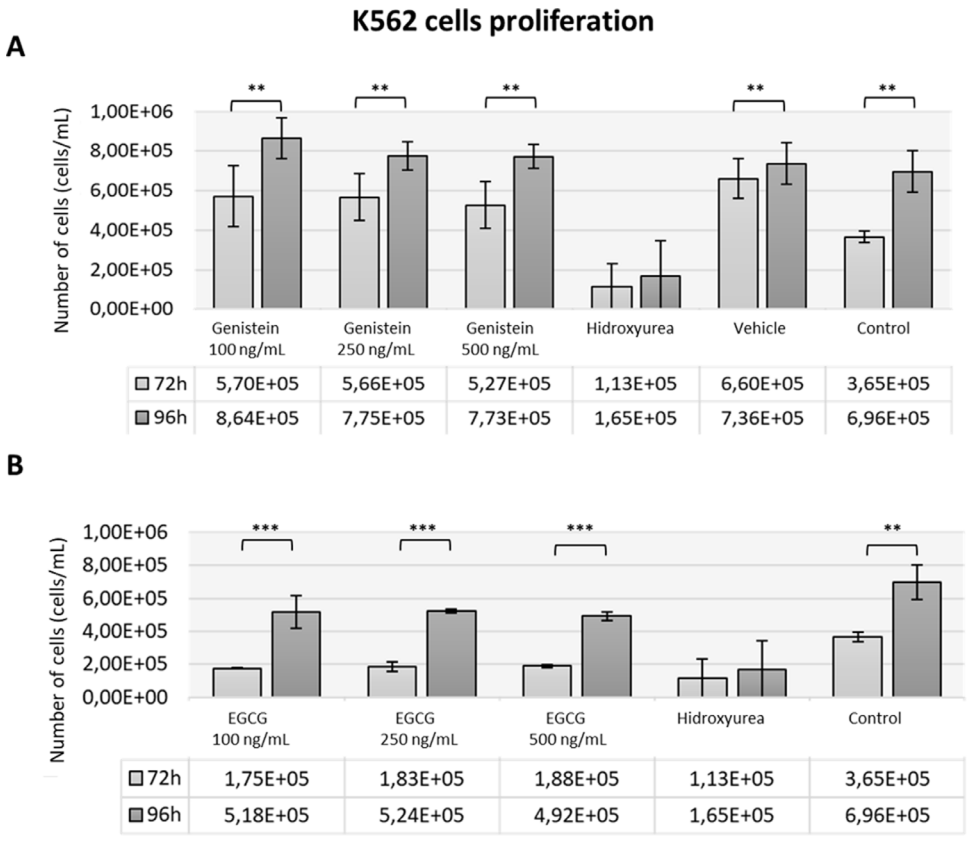

Fig. 1. GN (A) and EGCG (B) effect on K562 cells proliferation. Proliferation of K562 cells after 72 h and 96 h culture in control (control medium), vehicle (control media supplemented with 500 ng/mL DMSO), 25 μg/mL HU (positive control) and GN and ECGC at final concentrations of 100, 250 and 500 ng/mL. Initial cell density of 1×10^4 cells/mlL. Cultivation and viability assays were performed simultaneously for all growth conditions. Results are presented as the number of viable cells per mL ± standard deviation. $^{*}P < 0.05$ and $^{**}P < 0.01$ for Student's *t*-test.

(to the EGCG) and the cells exposed to vehicle DMSO (to the GN) were assessed using Student's *t*-test followed by one-way analysis of variance (ANOVA).

For *in vivo* assays, data were introduced and organized using the R software, through which all the statistical treatment was carried out. Shapiro-Wilk test was applied to the differences between each variable in T0 and T90. Both non-parametric (sign test) and parametric (*t*-test) for paired samples were used.

All the results are presented as means ± standard deviation. *P*-Values < 0.05 were considered statistically significant.

3. Results

3.1. EGCG and GN impact cellular metabolism and proliferation with no effects on cell viability

Cell proliferation data showed no significant differences in dose-dependent proliferation (with increasing concentrations) to GN exposure ($P > 0.05$). However, significant results ($P = 0.002$) regarding time-dependent exposure were observed as after 96 h of exposure, average proliferation rates for 100 ng/mL, 250 ng/mL and 500 ng/mL GN were 8.64×10^5 cells/mL, 7.75×10^5 cells/mL, and 7.73×10^5cells/mL, respectively. Maximal stimulation of K562 was observed at a GN concentration of 100 ng/mL after 96 h of exposure: 8.64×10^5 cells/mL (Fig. 1).

Regarding EGCG effects, no significant dose-dependent effects (for equal exposure times) between each treatment were observed, however time-dependent differences (for each dosage) were significant ($P \leq 0.001$), with an increased cell concentration at 96 h compared to 72 h: after 96 h of culture, the average proliferation rates for 100 ng/mL, 250 ng/mL and 500 ng/mL EGCG were 5.18×10^5 cells/mL, 5.24×10^5 cells/mL, and 4.92×10^5 cells/mL, respectively (Fig. 1).

High cell proliferation and a time-dependent response were also observed in the untreated control and vehicle solution with no significant effects in relation to EGCG and GN exposed cells.

On the other hand, HU inhibited almost 50% of cell proliferation at both measured time points in relation to control, which is compatible with cytostatic effects at the studied concentration (Fig. 1).

Moreover, potential cytotoxic and genotoxic effects of GN and EGCG exposure on K562 cells analyzed by Trypan-Blue exclusion assay showed no significant differences in cellular viability associated with dose and time exposure (98% of viability for 100 ng/mL, 250 ng/mL and 500 ng/mL at 72 h and 96 h for GN; 72 h ≥ 97% and 96 h = 100% for EGCG). The negative control at 96 h presented the highest cell viability rate (99% ± 1, 5%). Vehicle solution also demonstrates no influence on cellular viability, whereas HU effects, although not significant, were indicative of a viability decrease (96% ± 2.6%).

Cellular viability measurements based on cellular metabolic capacity through the Cell-Titer Blue assay method demonstrated that GN and EGCG had no effects after 72 h of exposure (Fig. 2A), whereas after 96 h of 250 and 500 ng/mL EGCG exposure increased cell metabolic activity was reported. Interestingly, 96 h of 100 ng/mL GN exposure resulted in a decrease of the parameter in relation to control (Fig. 2B). Vehicle solution did not affect cellular metabolic activity at any time point, whereas HU effects exposure resulted in altered cellular viability/metabolism with time divergent effects.

3.2. Transcription levels of HbF regulators decrease after GN and EGCG exposure with dose and time dependent divergent effects

GN concentrations evaluated at both exposure times induced the downregulation of *BCL11A* mRNA levels (Fig. 3). GN downregulation was significant at 72 h for the lowest (100 ng/mL $P = 0.038$) and highest (500 ng/mL $P = 0.003$) concentrations assayed (Fig. 3A) which resulted in a U-shaped dose response curve. Moreover, increased exposure (96 h) GN at 250 ng/mL induced a significant downregulation of *BCL11A* ($P = 0.003$), non-observable at the other concentrations for this time point (Fig. 3B) consistent with an inverted U-shaped dose response curve. Regarding EGCG exposure effects, we observed a severe decrease in *BCL11A* mRNA levels after 72 h of 100 ng/mL EGCG ($P < 0.001$) (Fig. 3C), similar to that obtained after 96 h of HU exposure (Fig. 3B,

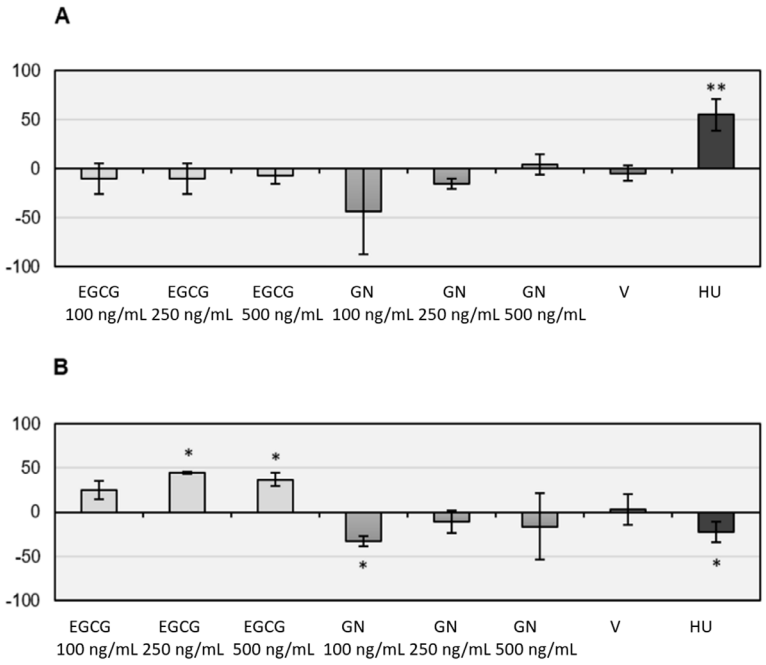

Fig. 2. Cell viability after 72 h (A) and 96 h (B) of control (control medium), vehicle (control media supplemented with 500 ng/mL DMSO), 25 μg/mL HU (positive control) ECGC and GN at final concentrations of 100, 250 and 500 ng/mL. Results are presented as percentage of variation over control, $^{*}P < 0.05$ and $^{**}P < 0.01$ for Student's *t*-test in relation to control.

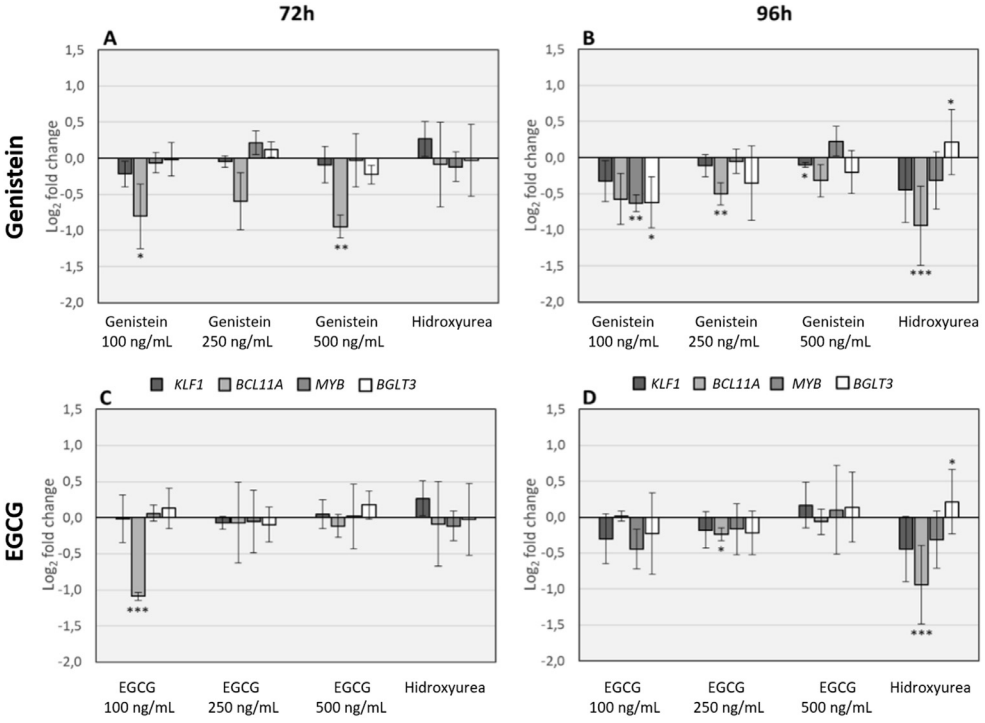

Fig. 3. qRT-PCR analysis in K562 cells after exposure to three different concentrations of Genistein (A; B) and EGCG (C; D) (100, 250 and 500 ng/mL), for 72h (A; C) and 96 h (B; D). Data represent the relative expression for the genes *KLF1*, *BCL11A*, *MYB* and *BGLT3*. GAPDH was used for normalization. (ΔΔCt values were $\log_2$-transformed). Error bars represent the standard deviation between two independent treatments and three qRT-PCR replicates. Significant statistical values, which were compared with the vehicle (DMSO) for Genistein and control for EGCG and calculated with Student's *t*-test, are illustrated as: $^{*}P < 0.05$ and $^{**}P < 0.01$.

D). After 96 h of EGCG exposure, significant effects were only reported for 250 ng/mL ($P = 0.041$) (Fig. 3D).

Concerning *KLF1* relative expression, our data reported a significant decrease in expression ($P = 0.028$) after 96 h of 500 ng/mL GN exposure (time and dose dependent (Fig. 3B). No significant effects were observed after EGCG or HU exposure at any time points (Fig. 3A, C, D).

Interestingly, the HbF regulator genes *MYB* and the noncoding *BGLT3* were exceptionally downregulated after exposure to 100 ng/mL of GN for 96h ($P = 0.002$ and $P = 0.017$, respectively) (Fig. 3B).

3.3. Globin expression levels are differentially affected by GN and EGCG exposure

GN exposure particularly affected globin gene expression at lower dosages, with downregulation of mRNA levels of *HBA* at 250 ng/mL after 72 h ($P = 0.006$) (Fig. 4A) and 100 ng/mL after 96 h of exposure ($P < 0.001$) accompanied by *HBG2* ($P = 0.001$) (Fig. 4B).

On the other hand, EGCG effects on globin mRNA levels were observed for *HBB* and *HBG1* also with increased effect at lower exposure concentrations (Fig. 4C, D). A significant downregulation of *HBB* levels was reported at 72 h time point for the lower and intermediate tested

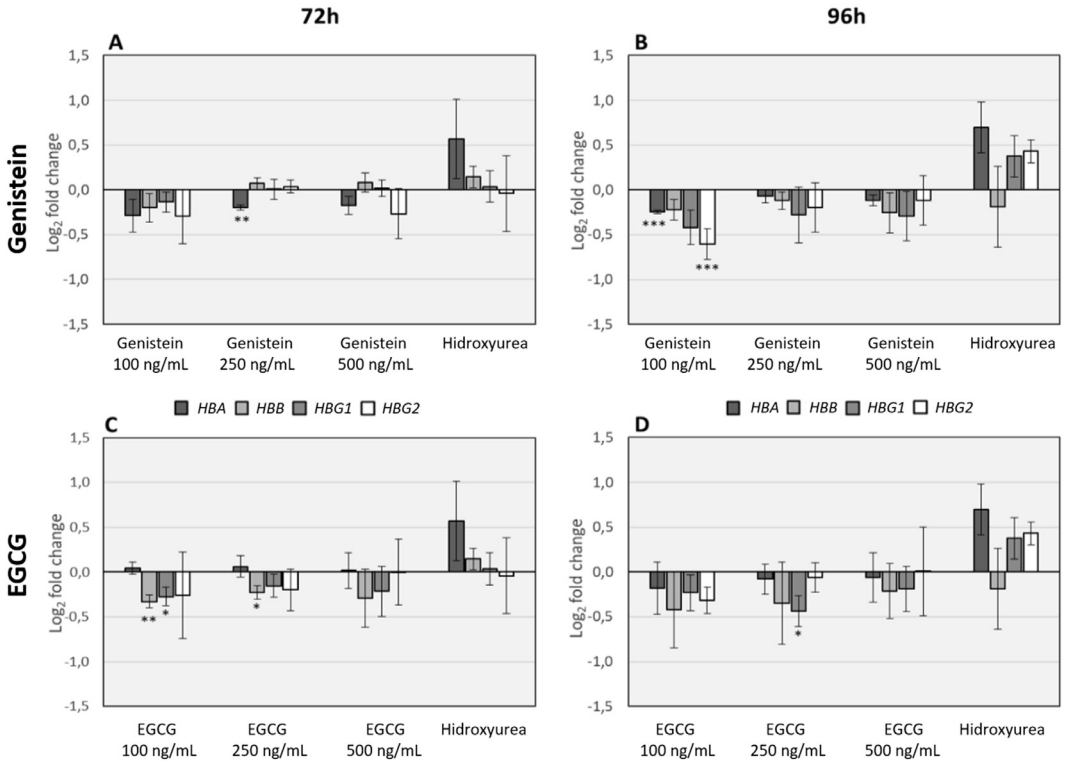

Fig. 4. qRT-PCR analysis in K562 cells after exposure to three different concentrations of Genistein (A; B) and EGCG (C; D) (100, 250 and 500 ng/mL), for 72 h (A; C) and 96 h (B; D). Data represent the relative expression for the globin genes, namely *HBA*, *HBB, HBG1* and *HBG2*. GAPDH was used for normalization. (ΔΔCt values were $\log_2$-transformed). Error bars represent the standard deviation between two independent treatments and three qRT-PCR replicates. Significant statistical values, which were compared with the vehicle (DMSO) for Genistein and control for EGCG and calculated with Student's *t*-test, are illustrated as: $^{*}P < 0.05$ and $^{**}P < 0.01$.

concentrations of 100 ng/mL ($P = 0.003$) and 250 ng/mL ($P = 0.015$) (Fig. 4C). Additionally, mRNA levels of *HBG1* at 72 h presented a downregulation with the concentration of 100 ng/mL ($P = 0.027$) and at 96 h of exposure, this gene was the only one with a significant downregulation ($P = 0.036$) (Fig. 4D). HU exposure did not significantly affect *HBA, HBB, HBG1 or HBG2* mRNA levels at any measured time point (Fig. 4).

3.4. Epigenetic modulators expression is altered by GN and EGCG exposure

GN exposure upregulated the expression of *DNMT1* significantly at all concentrations after 72 h, while only the two lowest concentrations managed to affect *DNMT3A* expression after this time and the highest dose made an impact in *DNMT3B* transcription (Fig. 5A). After 96 h of exposure, GN at 500 ng/mL was the only treatment that managed to affect *DNMT*, namely downregulating *DNMT1* ($P = 0.017$) (Fig. 5B).

Moreover, the gene *DNMT3A* was the only one with a significant upregulation for EGCG at 250 ng/mL after 72 h (Fig. 5C). At 96 h, EGCG did not significantly affect *DNMT1, DNMT3A* and *DNMT3B* mRNA levels at any measured dose points (Fig. 5D). HU at 25 μg/mL only upregulated *DNM3A* with statistical impact at 72 h ($P = 0.0006$) and 96 h ($P = 0.049$).

Exposure to GN after 72 h managed to upregulate *HDAC1* at 100 ng/mL and downregulate *HDAC8* ($P = 0.008$), this last one in greater levels than HU (Fig. 6A). At this time-exposure and the highest concentration (500 ng/mL), GN resulted in *HDAC1, HDAC3* and *HDAC8* downregulation ($P < 0.05$). Curiously, these last two genes suffered a greater modulation after cellular treatment with GN than with HU. Downregulation of *HDAC8* with GN after 72 h at the lowest and highest dose is consistent with a U-shaped dose response curve.

After 96 h of exposure, GN exposure resulted in upregulated *HDAC3* at 250 ng/mL ($P = 0.001$), while the highest dose upregulated *HDAC2* ($P = 0.037$) and simultaneously downregulated *HDAC8* ($P = 0.001$) (Fig. 6B).

Cellular exposure to EGCG during 72 h only managed to upregulate significantly the expression of *HDAC8* at the lowest dose ($P = 0.014$) (Fig. 6C). However, after 96 h, exposure to the lowest dose results in upregulation of *HDAC2* ($P = 0.002$) in greater levels than HU and the highest dose also downregulated this gene transcription ($P = 0.019$) (Fig. 6D).

After 72 h, HU exposure induced a downregulation of *HDAC8* ($P = 0.040$), while after 96 h it further managed to downregulate *HDAC1* ($P = 0.0001$).

3.5. EGCG administration affects hematological parameters and increases HbF levels in vivo

In this study where enrolled 30 volunteers which included 10 males and 20 females with ages range [19–43; average 27.1 years], weight range [45–108; average 65 kg] and height range of [155–195; average 170. 71 cm]. Out of the 30 participants, 4 were excluded from the statistical analysis due to potential induced bias in the results. Two participants were excluded due to a contracted viral infection during the study, and the other two for incomplete adhesion to capsule intake, since EGCG decreases to residual levels after 24 h (Mereles and Hunstein, 2011).

Considering Shapiro-Wilk tests, some of the differences between T0 and T90 do not verify the normality assumption ($P < 0.05$). Therefore, for the sake of consistency, the non-parametric sign test was used, which demonstrated statistical evidence to affirm that, at the established significance level, there is a significant difference between T0 and T90 in relation to the variables: hemoglobin, hematocrit, mean global volume, reticulocyte hemoglobin of fetal hemoglobin and total red blood cells. The parametric (*t*-test) for paired samples also confirmed the statistical significance of the same variables between T0 and T90, as demonstrated in Table 2.

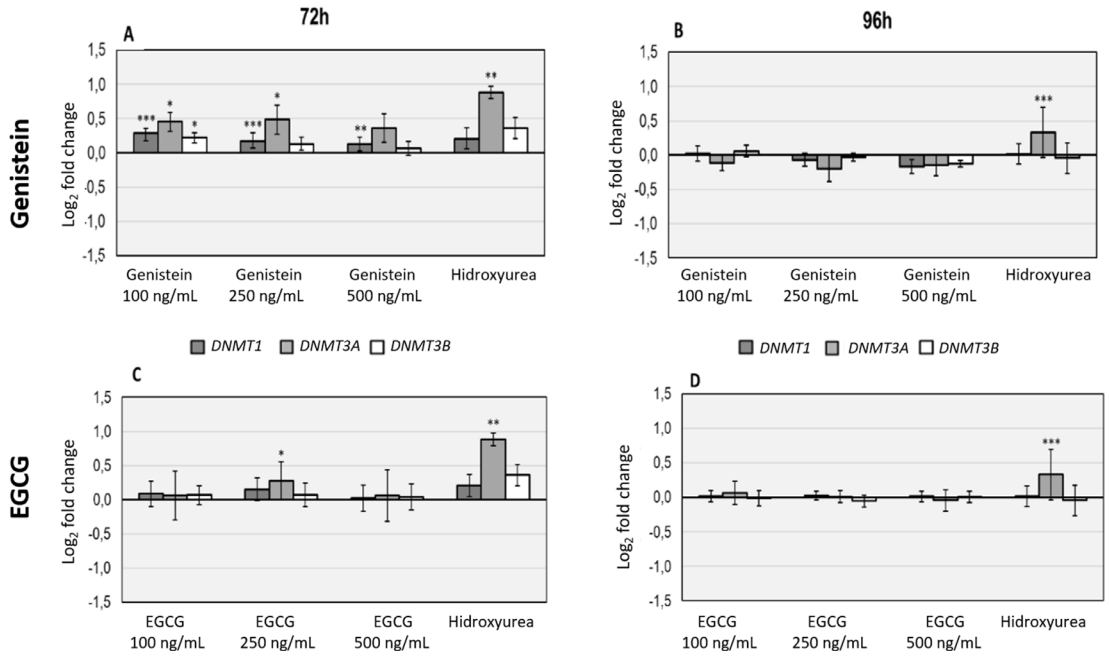

Fig. 5. qRT-PCR analysis in K562 cells after exposure to three different concentrations of Genistein (A; B) and EGCG (C; D) (100, 250 and 500 ng/mL), for 72 h (A; C) and 96 h (B; D). Data represent the relative expression for the DNA methyltransferase genes, namely *DNMT1, DNMT3A* and *DNMT3B*. GAPDH was used for normalization. (ΔΔCt values were $\log_2$-transformed). Error bars represent the standard deviation between two independent treatments and three qRT-PCR replicates. Significant statistical values, which were compared with the vehicle (DMSO) for Genistein and control for EGCG and calculated with Student's *t*-test, are illustrated as: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

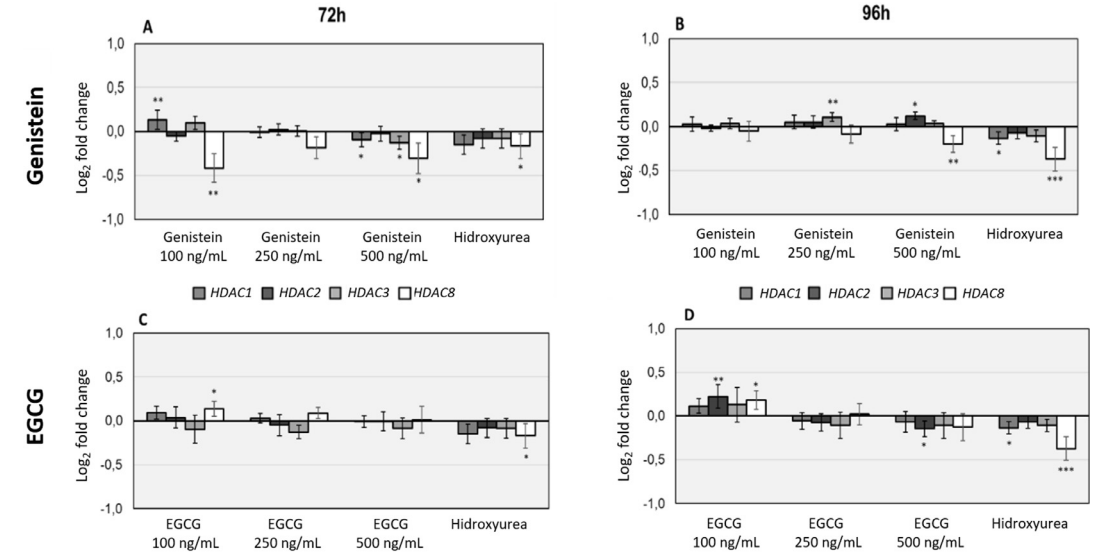

Fig. 6. qRT-PCR analysis in K562 cells after exposure to three different concentrations of Genistein (A; B) and EGCG (C; D) (100, 250 and 500 ng/mL), for 72 h (A; C) and 96 h (B; D). Data represent the relative expression for the histone deacetylase genes, namely *HDAC1*, *HDAC2*, *HDAC3* and *HDAC8*. *GAPDH* was used for normalization. (ΔΔCt values were $\log_2$-transformed). Error bars represent the standard deviation between two independent treatments and three qRT-PCR replicates. Significant statistical values, which were compared with the vehicle (DMSO) for Genistein and control for EGCG and calculated with Student's t-test, are illustrated as: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

4. Discussion

The development of new compounds capable of HbF induction with high efficiency and lower toxicity than the currently available therapeutic agents are the focus of intense research, particularly due to the high prevalence and incidence of these diseases worldwide. Additionally, the inability of populations in highly endemic developing countries to access proper medication and sustain the high costs of clinical management leads to the necessity to develop new therapeutic approaches with exceedingly accessible compounds at particularly low costs.

Here we performed an assessment of EGCG and GN, two compounds that trigger p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway (Cui et al., 2014; Shu et al., 2018) as HU (Chou et al., 2015), as a potential to affect transcriptional expression of globin and HbF regulators/silencer genes at 100, 250 and 500 ng/mL and evaluate, *in vivo* the effects of 225 mg EGCG intake during 90 days in hematological parameters.

Table 2
Complete blood count analysis conducted at T0 and T90, respectively and its corresponding average and standard deviation values.

Hematological parameters	T0		T90		*P*-value
	Mean	Standard Deviation	Mean	Standard Deviation	2 tail (Paired)
Erythrocytes (10^{12}/L)	4.573	0.313	4.693	0.410	0.017
Hemoglobin (g/dL)	13.796	1.182	14.290	1.348	0.001
Hematocrit (%)	40.310	2.942	41.930	3.942	< 0.001
Mean cell volume (fL)	88.100	3.417	89.366	3.326	< 0.001
Mean Corpuscular Hemoglobin (pg)	30.100	1.807	30.433	1.406	0.105
Mean corpuscular hemoglobin conc. (g/dL)	34.233	0.971	34.366	1.033	0.536
Red Cell Distribution Width (RDW %)	12.946	0.723	12.823	0.659	0.089
Reticulocytes (%)	1.587	0.398	1.446	0.445	0.092
Absolute value (10^9/L)	72.300	18.474	68.833	20.122	0.323
Reticulocyte hemoglobin content (pg)	31.966	2.042	33.233	1.695	< 0.001
Fetal hemoglobin (HbF) level (%)	0.396	0.140	0.450	0.157	< 0.001
Leukocytes (10^9/L)	7.081	1.522	7.483	1.429	0.097
Neutrophils (10^9/L)	4.282	1.197	4.585	1.185	0.149
Neutrophils (%)	59.936	7.659	61.083	7.939	0.463
Eosinophils (10^9/L)	0.185	0.140	0.176	0.100	0.664
Eosinophils (%)	2.677	1.994	2.380	1.382	0.260
Basophils (10^9/L)	0.085	0.310	0.030	0.006	0.340
Basophils (%)	1.366	5.294	0.400	0.000	0.325
Lymphocytes (10^9/L)	2.201	0.687	2.364	0.704	0.208
Lymphocytes (%)	32.173	6.784	31.723	7.637	0.747
Monocytes (10^9/L)	0.334	0.080	0.327	0.094	0.669
Monocytes (%)	4.813	1.186	4.413	1.060	0.081
Platelets (10^9/L)	243.100	53.880	251.366	54.868	0.172

The *P*-value of the *t*-test comparing T90 and T0 are represented, being highlighted in bold *P*-values lower than 5%.

Human clinical pharmacokinetic studies show that the effective plasma level of total genistein is in the micromolar range while the genistein aglycone is in a hundred nanomolar range *in vivo*, and plasma concentration of 155 ng/mL has been detected in human samples associated with normal dietary intake, reviewed in (Yang et al., 2012). However, after supplementation of purified isoflavones, blood concentration may increase, which leads to the selection of the assayed dosages (100, 250 and 500 ng/mL) (Raschke et al., 2006) and steady-state plasma concentration would be more readily maintained by repeated ingestions throughout the day (Setchell et al., 2003). On the other hand, human clinical trials demonstrated that 40 mg and 800 mg of EGCG intake (Tea, 2009), results in peak serum concentrations in the range of 100–400 ng/mL (Chow et al., 2003) with no reported severe secondary effects. Therefore, the concentrations of the compounds in this study are considered safe and mimic the *in vivo* cellular exposure of GN and EGCG after supplementation.

Our data demonstrated that after 72 h of exposure EGCG and GN do not affect cellular metabolic capacity. However, after 96 h of exposure, we reported divergent effects, whereas EGCG exposure resulted in increased metabolic capacity in a dose dependent manner, and GN at the lowest concentration tested results in decreased cell metabolism. Nevertheless, no negative effect on cellular proliferation or viability was reported through trypan blue exclusion assay, and interestingly the higher proliferation level of K562 was observed at a GN concentration of 100 ng/mL after 96 h of exposure inversely to cellular metabolic activity.

Previously published results indicated that GN biological effects diverge in relation to doses applied (Russo et al., 2016), including metabolic alterations and decreased cellular proliferation (Ganai and Farooqi, 2015). Additionally, EGCG potential to affect cellular metabolism has also been evaluated and described in a dose dependent manner (Chakrawarti et al., 2016) and divergent effects regarding cellular proliferation in normal and cancer cells associated with decreased cell division (Ni et al., 2018). Our results also suggest that GN endorses a higher effect on cell proliferation compared to EGCG, which is in concordance with previously published studies in hepatic stellate cells, T6/GFAP-lacZ cells (Zhang and Zhuo, 2006) and DU145 cells (Bhatia and Agarwal, 2001).

Data observed in this study support GN and EGCG (at studied concentrations) potential to be used as possible pharmacological agents, showing no cytostatic effects, which is a significant advantage over other known inducers, such as HU.

Furthermore, considering that *KLF1, BCL11A, MYB and BGLT3* expression is crucial for HbF silencing (Sankaran, 2011), we used qRT-PCR to assess GN and EGCG exposure effects on these genes transcriptional levels.

GN exposure resulted in downregulation of *BCL11A* mRNA levels at 72 h for 100 ng/mL and 500 ng/mL concentrations and at 96 h at 250 ng/mL doses. Our results clearly demonstrated that GN effects on K562 cells follow a non-monotonic dose-response (NMDR) and time divergent response, which is common in hormones as well as endocrine-disrupting chemicals (EDCs) such as phytoestrogens (Vandenberg et al., 2012). In agreement with our results, *BCL11A* downregulation induced by GN has also been reported in LNCaP cells (Merchant et al., 2012). Interestingly, we also observed a severe downregulation of *BCL11A* mRNA levels after 72 h of EGCG exposure at the lowest concentration and at 96 h time point a more subtle but significant effect was observed for the intermediate dosage (250 ng/mL), which is also suggestive of a NMDR effect. Recent evidence demonstrated that EGCG is able to target estrogen receptors, particularly ERα36 (an isoform of estrogen receptor alpha (ERα)) (Chen et al., 2019) and ERRα (Shu et al., 2018). It suggests that ERα36 and other estrogen receptors might be efficient targets of EGCG. Furthermore, exposure to HU also resulted in downregulation of *BCL11A* mRNA levels, which is in agreement with previous results (Pule et al., 2016). Our results advocate that both GN and EGCG may have a specific molecular target for *BCL11A* but not *KLF1,* as no analogous effects were observed for *KLF1* mRNA levels. In fact, erythroid-specific *BCL11A* downregulation, without altering the pleiotropic transcription regulator *KLF1* levels, has already been described and associated with strong HbF inducers such as Pomalidomide, an FDA-approved third-generation immunomodulatory drug, originally developed for the treatment of myeloma, which induces HbF production by promoting erythropoiesis both *in vitro* and *in vivo* (Appiah-Kubi et al., 2013; Dulmovits et al., 2016). Despite the observed erythroid-specific *BCL11A* downregulation, no effects were observed regarding increased *HBG1* expression levels. However, considering that exposure to HU, which is currently utilized for the treatment of SCD patients (Field and Nathan, 2014) due to *HBG1* gene induction and reduced *HBB* gene expression (Chou et al., 2015), also did not result in augmented *HBG1 or HBG2* transcription or diminished *HBB* gene expression, we postulated that exposure should be prolonged in further studies.

On the other hand, recently published data on epigenetic editing of *BGLT3* through CRISPR/Cas9 proven is specific regulation of γ-globin genes (Ivaldi et al., 2018) which is in agreement with our results as *BGLT3* downregulation is accompanied by HBG2 in GN 100 ng/mL 96 h exposure, and the observed upregulation of *BGLT3* after HU exposure brings new insights regarding HU molecular mechanisms associated with its HbF inducer capacity.

It is also important to notice that our data demonstrated a significant downregulation of *HBB* levels after EGCG accompanied by *BCL11A* downregulation at 100 ng/mL, which is particularly promising considering that decreased *HBB* gene expression is one of the effects of HU HbF inducer (Chou et al., 2015).

Overall transcriptional data demonstrated that EGCG and GN appear to present HbF inducer potential through divergent signaling pathways considering that GN affected *HBA* and *HBG2* expression whereas EGCG altered *HBB* and *HBG1* transcriptional levels and the regulator genes *KLF1, MYB* and *BGLT3* were only affected by GN. Nevertheless, the key transcription factor silencer *BCL11A* is affected by both compounds.

Furthermore, EGCG and GN are recognized as epigenetic modulators and so, in this work, qRT-PCR was also performed to analyze the mRNA levels of key epigenetic modulators, namely *DNMT1, DNMT3A, DNMT3B* and all the class I *HDAC*.

EGCG only affected mRNA levels of *DNMT3A* at 250 ng/mL and 72 h of time-exposure, but no other time or dose made a statistical impact. Previous studies show that this natural compound is able to alter methylation status, decreasing *DNMT3B* expression in HeLa cells (EGCG 25 μM, 72 h treatment) (Khan et al., 2015), and of all *DNMT* genes in Jurkat cells (10 μM, 48 h treatment)(Wong et al., 2011) and in A431 cells (10–20 μg/mL, 72–144 h treatment) (Nandakumar et al., 2011). Not only is cell lineage different from the one used in this study, but also concentrations of EGCG were much higher, which might explain the significant impact in the mRNA levels that were not obtained in our study. Still, EGCG's ability to inhibit DNA methylation is controversial because it does not always have an effect on cellular methylation. As so, our results are concordant with previously obtained studies where EGCG did not inhibit DNA methylation in T24, HT29 and PCR3 cells (Chuang et al., 2005) and also in TK6, Jurkat and KG-1 cells (Stresemann et al., 2006).

Although it did not appear to change methylation extensively, EGCG had effects on deacetylation, while results suggest a NMDR effect which was previously reported for this compound. Cellular exposure for both exposure times to 100 ng/mL managed to upregulate *HDAC8,* while at 96 h there were divergent effects since the lowest dose upregulated *HDAC2* and the highest dose downregulated this gene in greater levels than HU. The enzyme *HDAC2* has been implied in the HbF switch as a repressor of the *HBG* genes (Bradner et al., 2010), so inhibition of their expression by EGCG demonstrates its ability as a potential HbF inducer.

As for GN, after 72 h exposure at the highest dose, we reported lower mRNA levels of *HDAC3* to a greater extent than the one obtained for HU and at the same time, also making an impact downregulating *BCL11A*. *HDAC3* has been implied in the switch of expression from HbF to HbA since it is knockdown was sufficient to significantly upregulate γ-globin genes, and after 96 h of exposure GN downregulated the expression of *DNMT1* at the highest dose (500 ng/mL). DNMT1 has been identified as a cooperator of both HbF suppressors BCL11A and MYB (also repressing γ-globin genes expression) (Roosjen et al., 2014), so its downregulation by GN in greater levels compared to the one obtained for HU at the same time demonstrates its potential as a HbF inducer. Our results are concordant with previous studies that GN has the ability to decrease global methylation and expression of *DNMT1* (Xie et al., 2014).

Moreover, our data also demonstrates HU potential to affect epigenetic modulators. HU exposure induced upregulation of *DNMT3A* in a time dependent manner. Additionally, for the shortest exposure time, HU appeared to cause downregulation of *HDAC8*, further modulating mRNA levels of *HDAC1* at 96 h. HU seems to have a significant effect on methylation at 72 h and in deacetylation at 96 h, suggesting that different signaling pathways might be involved in his epigenetic modulatory mechanism. Interestingly, after 72 h of time exposure, the lower concentration of EGCG upregulated *HDAC8* and also downregulation of *BCL11A* that was similar to the one caused by HU. Furthermore, GN exposure at the shortest time with the lower and highest dose (100 and 500 ng/mL) are associated with a downregulation *HDAC8* and concomitant decrease in mRNA levels of *BCL11A*. As for HU, at both exposure times, it causes downregulation of *HDAC8* with downregulation of *BCL11A* at 96 h. The enzyme *HDAC8* does not have an established role in HbF regulation. However, our results suggest that it might be involved in this process since it appears to be associated with HbF regulators effects at all applied exposures (EGCG, GN and HU).

Furthermore, even though GN and EGCG concentrations tested in this study are considered safe (for cell survival) and mimic plasma concentrations obtained after oral intake (Klein and King, 2007; Wu et al., 2012), considering the results obtained in cellular metabolic activity and the fact that GN is a phytoestrogen with associated NMDR (Russo et al., 2016), to ensure participants safety we performed the *in vivo* study with supplementation of only EGCG (225 mg).

Our *in vivo* results indicated statistical evidence to affirm differences between T0 and T90 in relation to the following variables: total number of red blood cells, amount of hemoglobin, hematocrit percentage, reticulocyte hemoglobin count, fetal hemoglobin amount. It is relevant to notice that in a complete blood count; only the hemoglobin associated parameters were affected after 90 days of EGCG oral exposure (Aygun et al., 2002).

On the other hand, regarding leukogram data, we reported a positive, however not statistically significant, variation of the mean values in total leukocytes, neutrophils and lymphocytes, between T0 and T90, which is in agreement with the modulatory effect of EGCG on the immune system (Paea and Wu, 2013), and is particularly relevant considering the mortality rate of SDC patients associated with infections (Reeves et al., 2019).

Overall, our results demonstrate the potential of natural compounds, particularly EGCG, for the treatment of SCD patients through the pharmacological reactivation of HbF. Further studies regarding mediated epigenetic effects and p38 MAPK signaling pathway activation must be performed in order to understand better the molecular mechanisms underlying HbF induction and its potential use as a new therapy approach.

5. Conclusion

The worldwide impact of the high prevalence of β-hemoglobinopathies, particularly in developing countries, combined with the low efficacy and adverse side effects of existing therapies, created an urge to investigate and develop new, less harmful, and preferably low-cost therapeutic approaches, including pharmacological agents of HbF induction. This study demonstrated the potential of natural compounds EGCG and GN to affect transcriptional expression of HbF silencer genes and the *in vivo* modulator effect of EGCG consumption in hematopoiesis and HbF. Moreover, we also bring new insights regarding HU molecular mechanisms associated with its HbF induction capacity.

Our data advocates HbF pharmacological reactivation potential of EGCG and GN, which should be considered and evaluated in further studies as new alternative approaches for β-hemoglobinopathies therapy.

Ethical Approval

This work is included in a project from the Instituto Politécnico de Lisboa accepted in Escola Superior de Tecnologia da Saúde ethical council ref: CE-ESTeSL-N°.18-2019 and CE-ESTeSL-N°.10-2019. All volunteers provided a signed written informed consent before enrolment in

the study in accordance with the Helsinki Declaration and Oviedo Convention and in Agreement with the Portuguese law n° 58/2019 de 8 de agosto regarding data protection. All volunteers provided a signed written informed consent before enrolment in the study in accordance with the Helsinki Declaration and Oviedo Convention and in Agreement with the Portuguese law n° 58/2019 de 8 de agosto regarding data protection.

Data Availability

All data and material will be available as requested.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

CRediT authorship contribution statement

Edna Ribeiro: Visualization, Writing – original draft, Supervision, Writing – review & editing, Validation. **Mariana Delgadinho:** Methodology, Writing – original draft, Writing – review & editing, Validation. **Elisabete Matos:** Methodology, Writing – review & editing, Validation. **Raquel Santos:** Methodology, Writing – review & editing, Validation. **Daniela Sousa:** Methodology, Writing – review & editing, Validation. **Heloísa Galante:** Formal analysis, Writing – review & editing, Validation. **Miguel Brito:** Visualization, Writing – original draft, Supervision, Writing – review & editing, Validation.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ccmp.2022.100034.

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