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The investigation of resveratrol and analogs as potential inducers of fetal hemoglobin

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ABSTRACT

Beta-thalassemia, is a hemoglobinopathy characterized by reduced beta-globin chain synthesis, leading to imbalanced globin chain production, ineffective erythropoiesis and anemia. Increasing gamma-globin gene expression is a promising therapeutic approach as it reduces this imbalance by combining with the excess alpha globin chains and producing fetal hemoglobin (HbF). Furthermore, increased iron absorption and repeated blood transfusions lead to iron overload and tissue damage secondary to reactive oxygen species. Compounds exhibiting both antioxidant and HbF inducing activities are, therefore, highly desirable therapeutic agents. Resveratrol, a natural phytoalexin, combines these two activities but is also cytotoxic. Nine hydroxystilbenic resveratrol derivatives were synthesized in an attempt to identify compounds that retain the HbF-inducing and antioxidant activities of resveratrol but exhibit reduced cytotoxicity. Three derivatives (P1, P4 and P11) exhibited similar hemoglobin-inducing properties to resveratrol in K562 cells, however, only P11 showed reduced cytotoxicity. All three derivatives demonstrated variable HbF-inducing activity in primary erythroid progenitor cells from healthy donors. Resveratrol and P11 increased HbF induction significantly, with P11 having the highest activity. Additionally, P4 significantly increased progenitor numbers. A combinatorial treatment in K562 cells using resveratrol and decitabine resulted in a statistically significant increase in hemoglobin-inducing activity only above the level shown by resveratrol alone.

1. Introduction

Beta-thalassemia, a global health burden, is an inherited disorder of beta globin chain production. The disease is characterized by reduced synthesis of functional β-globin chains, leading to imbalanced globin chain ratio, ineffective erythropoiesis and anemia [1]. As a result, thalassaemic patients require regular blood transfusions with regular iron chelation therapy as a means of managing the disease. However, this treatment is not curative and still has substantial morbidity. A very promising therapeutic approach for β-thalassemia is the production of fetal hemoglobin (HbF) through pharmacological reactivation of endogenous gamma-globin genes as γ-globin can substitute for the absent or reduced adult β-globin [2,3]. The currently available HbF-inducing chemical agents have limited clinical application due to their moderate therapeutic properties and potential cytotoxic effects [4,5]. Therefore, identification of novel agents with higher HbF inducing activity and lower cytotoxicity has been one of the major challenges over the past few years.

Oxidative stress has been observed in various types of thalassemia as well as in other hereditary and acquired hemolytic anemias. This can be mainly attributed to iron overload due to increased iron absorption in the gastrointestinal tract [6], multiple blood transfusions as well as to increased intracellular denaturation of imbalanced hemoglobin subunits resulting in dissociation of heme from globin and iron from heme [7,8]. Labile non-transferrin bound iron can be involved in chemical reactions that generate reactive oxygen species (ROS) that affect various cellular components, particularly the cell membrane, damaging vital organs including the heart, liver and endocrine system [9]. In addition, endogenous antioxidant mechanisms are depleted due to the increased need to neutralize the oxidative stress [10]. Oral administration of vitamin E in transfusion-independent beta-thalassemia patients was shown to decrease ROS production and increase glutathione reductase levels in red blood cells [11]. Moreover, natural antioxidants such as curcuminoids [12] and fermented papaya preparation [13] were studied for their potential use towards elimination of antioxidant stress.

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Resveratrol is a natural phytoalexin found in a variety of human dietary products such as in the skin of red grapes, peanuts and red wine as well as in medicinal plants where it is produced in response to infection or other stresses. Resveratrol was originally isolated in 1940 from the root of *Veratrum grandiflorum*, a poisonous medicinal plant [14] known for its antimicrobial activity. However, it was not until the beginning of 1990 that research into resveratrol intensified due to evidence of the cardioprotective effects of red wine [15]. Since the 1990s, growing evidence from several studies has been accumulating showing resveratrol to have many biological activities including anti-inflammatory [16], anti-proliferative [17], chemopreventive [18,19], antioxidant [20,21] and lifespan enhancing activities [22–24], while having the ability to limit or prevent progression of cerebral ischemic injuries, cardiovascular injuries [25,26], cancer progression [11,18,27], arthritis [28,29], diabetes [30], neurodegernerative disorders [31,32] and a number of other aging-associated and stress resistance disorders. The mechanisms by which resveratrol is able to exert such a wide range of effects is not fully understood, but studies have led to the identification of a large number of direct targets for this compound. However, a number of its biological actions have been attributed to its antioxidant properties.

Apart from its antioxidant activities, resveratrol was found to inhibit ribonucleotide reductase in the same manner as hydroxyurea, the well known HbF inducer, as well as to promote erythroid differentiation [33]. Fibach et al. [34] were the first to show that resveratrol, in addition to its antioxidant activity, can also stimulate the expression of γ-globin genes and increase fetal hemoglobin production. In the current study, we investigated the ability of resveratrol and nine new hydroxystilbenic derivatives as potential HbF-reactivating agents as well as the combined effect of resveratrol and decitabine, another known HbF-inducer, as potential therapeutic approaches for β-thalassemia.

2. Material and methods

2.1. Materials

Resveratrol (3,4′,5-trihydroxystilbene) was purchased from Sigma (St Louis, USA). The nine resveratrol analogs were designed and synthesized by Perkins condensation following the procedure described elsewhere [35]. The purity of each compound (>97%) was controlled by HPLC.

2.2. Preparation of agents

Stock solutions of all compounds were prepared in 100% methanol and kept at −20 °C. The working solutions were prepared in 50% methanol. The chemical structures of the compounds tested are shown in Fig. 1.

2.3. Culture of human K562 cell line and analysis of cell differentiation

The human erythroleukemic cell line K562 was maintained in RPMI medium (Gibco, Invitrogen Inc., Paisley, UK) enriched with 10% FBS (Gibco, Invitrogen Inc., Paisley, UK), 0.6% Glutamine (Gibco, Invitrogen Inc., Paisley, UK) and 50 U/ml of Penicillin/Streptomycin (Gibco, Invtrogen Inc., Paisley, UK). The cells were kept at 37 °C with 5% CO_2 in a humified environment. Varying concentrations of each agent under investigation were added in 2×10^4 cells/ml. Cell survival measured by Trypan blue (Sigma, St. Louis, USA) and the Hb inducing ability of each agent measured by Benzidine staining were investigated for all derivatives after 5 days of treatment. Un-treated cells were used as negative control and cells treated with 150 μM Hydroxyurea as positive

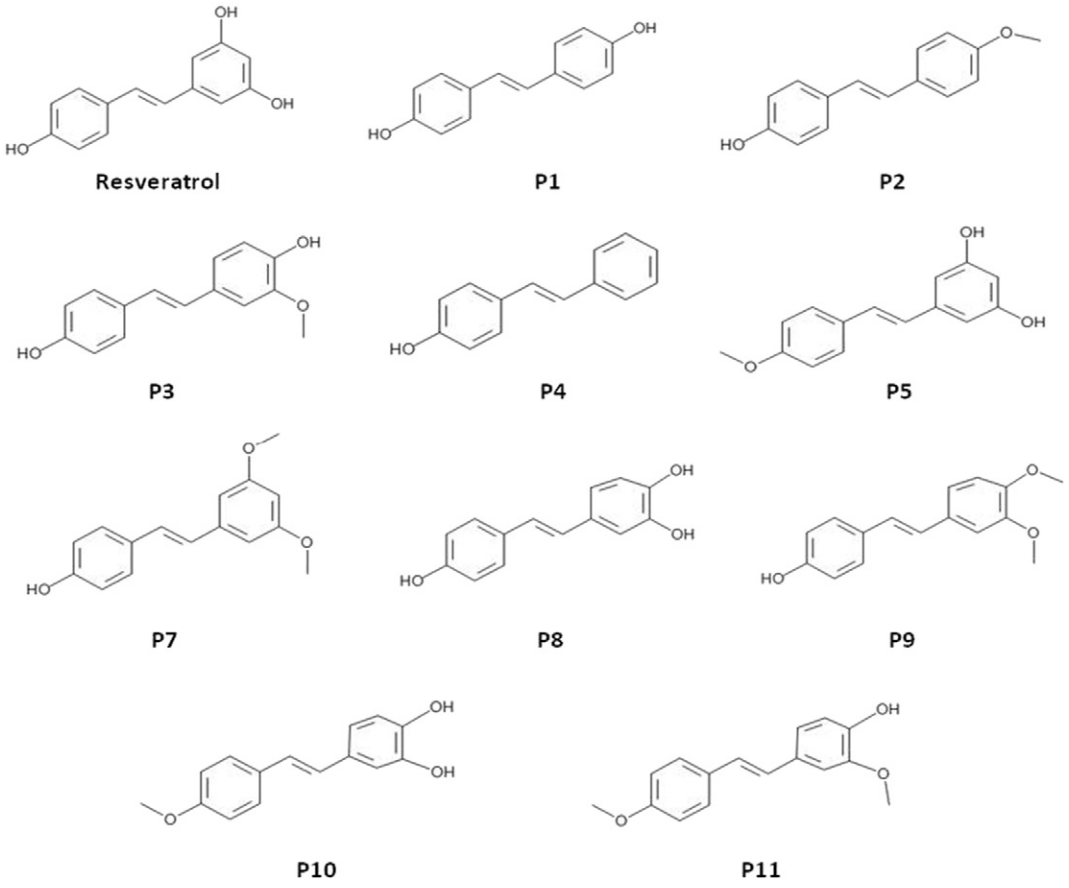

Fig. 1. Structures of the nine hydroxystilbenic derivativeanalogs to resveratrol along with resveratrol, the parent compound, which is commercially available.

control (Sigma Aldrich, St. Louis, USA). Triplicates of each culture condition studied were performed.

2.4. Benzidine test

Stock benzidine solution was prepared by the addition of 1 g benzidine dihydrochloride (Sigma Aldrich, St. Louis, USA) in 14.5 ml glacial acetic acid to 485.3 ml of distilled water. The working solution was prepared by the addition of 20 μl of 30% H_2O_2 to 1 ml of benzidine stock solution. Equal volumes of the working solution and cells are combined and allowed to stand at room temperature for 2–3 min. Hemoglobin (Hb) containing cells are stained blue and can be scored using a hemocytometer.

2.5. Primary human erythroid cultures from healthy donors

Human erythroid progenitor cells were cultured using the two phase liquid culture as previously described [36]. Briefly, mononuclear cells were isolated from buffy coats of healthy donors obtained from the Nicosia Blood bank by Lympholyte (Tebu-Bio, Le-Perray-en-Yvelines France) density gradient centrifugation separation. The layer of mononuclear cells was seeded in α-MEM (Sigma Aldrich, St. Louis, USA) supplemented with 10% Fetal Bovine serum (FBS) (Not heat inactivated) (Gibco, Invitrogen Inc., Paisley UK) 10% conditioned medium from the H5637 bladder carcinoma cell line and 1 μg/ml cyclosporin A (Sigma Aldrich, St. Louis, USA). On day 7 of incubation in Phase I, non-adherent cells were re-suspended in α-MEM (Sigma Aldrich, St. Louis, USA) supplemented with 5 U/ml/5 μg/ml penicillin/streptomycin, 10% FBS (Gibco, Invitrogen Inc., Paisley UK), 1 μg/ml cyclosporin A, 10% conditioned medium from cultures of H5637 bladder carcinoma cell line, 10 ng/ml stem cell factor (Gibco, Invitrogen Inc., Paisley UK) and 1 U/ml EPO. This constitutes the start of Phase II of the primary erythroid cultures.

2.6. Treatment of human primary erythroid progenitor cells

For the study of potential HbF inducers, compounds were added to the primary erythroid cell cultures on day 6 of Phase II. Human primary erythroid progenitor cell were seeded at ~1.5–2.0 × 10^6 cells/ml and different amounts of the compounds to be studied were added. Cells without any agent added (un-treated samples) were used as negative controls, and cells treated with 150 μM Hydroxyurea (Sigma Aldrich, St. Louis, USA) were used as positive controls. Cell counts and benzidine staining were performed on all samples after five days of treatment with the agents. HbF levels were determined by cation exchange high performance liquid chromatography (HPLC).

2.7. Cation exchange high performance liquid chromatography (HPLC)

The levels of HbF in primary erythroid cultures were determined by HPLC [37] using the Schimadzu Instrument (Kyoto, Japan) with DGUA5 degasser, LC-20AD pump, SIL-20AC HT autosampler, SPD-M20A PDA detectors and CTO-20A oven. Hemoglobins were separated on a PolyCat Atm 35 × 4.6 mm, 5 μm, 1000 A column (PolyLC Inc., Columbia, USA). The samples were eluted with a gradient of Bis-Tris-KCN-NaCl buffers and hemoglobins were detected at 417 nm.

3. Results

Initially resveratrol was screened *in vitro* in K562 cells. K562 cells are multipotential, hematopoietic, malignant cells that have the ability to reproducibly provide a large, uniform, population of cells that exhibit a stable and synchronized pattern of differentiation in the presence of chemical inducers such as hemin [38]. It is this innate ability to produce hemoglobin, their immortality and accessibility that renders K562 cells the most highly used model as a first line screen of potential agents. Screening of the agent in K562 cells showed resveratrol to be a highly active Hb-inducing agent resulting in a 5.7-fold increase in the number of hemoglobin containing cells and 50% cell survival relative to the untreated sample when used at 5 μM (Table 1). This was higher than the average effect observed with 150 μM hydroxyurea [39], the positive control compound which showed an average of 4.78-fold increase in the number of Hb-positive cells at a concentration 30-fold higher than resveratrol (Table 1). However, resveratrol was found to be slightly cytotoxic with cell survival reduced to ~35% at 10 μM relative to the untreated control. Therefore, the use of resveratrol might be limited due to this cytotoxicity. As a result, nine hydroxystilbenic resveratrol derivatives were designed and synthesized, with the aim of identifying an agent that retains this potent Hb inducing activity but has lower cytotoxicity than resveratrol. All nine derivatives had the same backbone structure as the parent compound but had small substitutions on the othro, meta and para positions of the two aromatic rings (Fig. 1). Initial screening of the nine derivatives was performed in K562 cells where the effect of a number of different concentrations of each derivative was investigated after five days of incubation with the agents (Supplementary). For each derivative, the concentration with the highest Hb inducing activity, while still maintaining a 40–50% cell survival, is shown in Table 1. Only three derivatives (P1, P4 and P11) exhibited Hb inducing activity comparable to the parent compound, with a fold increase in the percentage of Hb containing cells relative to the un-treated control of 3.8, 2.9 and 3.3 respectively. Among the latter three compounds, P11 had the lowest cytotoxicity while maintaining comparable hemoglobin-inducing activity to the rest of the compounds. This difference in cytotoxicity was apparent at concentrations of 5 μM and above (Fig. 2).

Derivatives P1, P4 and P11 along with resveratrol, were further studied in primary human erythroid cultures from healthy donors. HPLC analysis, which can detect specifically the levels of HbF in the sample, showed that individual cultures do not respond to the same extent to the each agent. Therefore, the cultures were divided into responders and non-responders with respect to HbF production following exposure to each test chemical. Cultures which responded to a chemical (at any test concentration) with an increase in HbF production of more than 10% were classed as responders to that chemical while the remainder of the cultures was classed as non-responders. Responders to resveratrol ($n = 5$) demonstrated a 33% increase in HbF levels at 1 μM of resveratrol, an effect that was statistically significant (p-value < 0.05). Similarly, responders to P11 ($n = 6$) demonstrated a 40 and 32% increase in HbF above the un-treated control at concentrations of 0.01 and 0.05 μM respectively. These increases were also statistically significant (p-value < 0.05). Interestingly, non-responders to both

Table 1
Investigation of the hemoglobin inducing ability (Benzidine stain) and cytotoxicity (Trypan blue) of each resveratrol derivative in the K562 cell line. The concentration of each agent resulting in approximately 50% cell survival and the corresponding effect in hemoglobin production is presented. 150 μM Hydroxyurea was used as a positive control in all experiments. The values shown represent the average of three experiments.

Agent	K562 cell line		
	Concentration (fiM)	Cell survival (% relative to uninduced control)	Benzidine stain (fold increase relative to un-induced control)
Un-treated	–	100	1
HU	150	38.9	4.78
PI	5	43	3.8
P2	5	48.8	2.3
P3	5	44	2.8
P4	5	48	2.9
P5	20	65.5	2.56
P7	20	41.2	2.86
P8	20	48.9	1.9
P9	5	51.7	1.9
Pll	10	52	3.3
Resveratrol	5	47.3	5.7

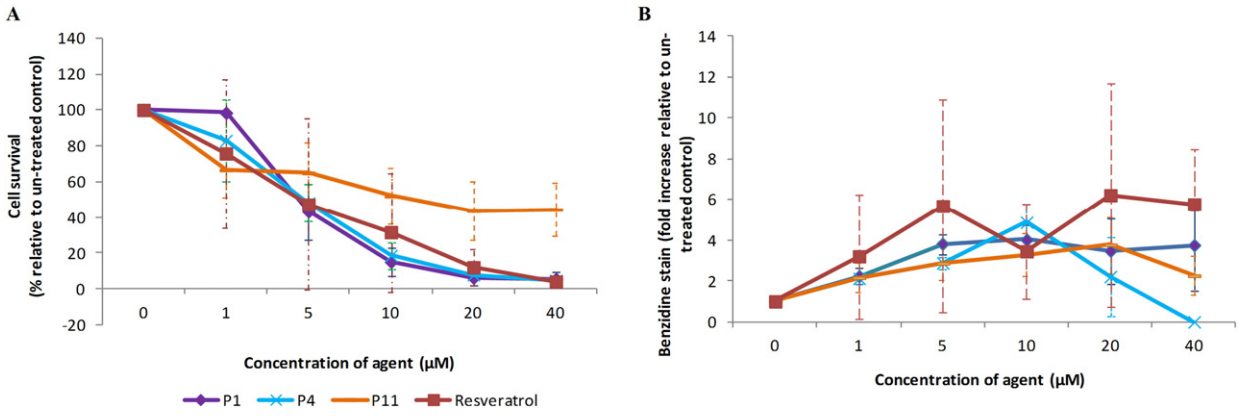

Fig. 2. Investigation of hemoglobin inducing activity (A) and cytotoxicity (B) of compounds P1, P4, P11 and resveratrol for a range of concentrations in K562 cells. P1, P4 and P11 showed hemoglobin induction levels similar to resveratrol, the parent compound, with P11 being the least cytotoxic. The results are the average of three experiments with error bars corresponding to the standard deviation.

agents (n = 7 for resveratrol, n = 7 for P11) demonstrated a significant reduction in HbF levels when compared to the un-treated controls (p-value < 0.05, paired t-test) at 0.01 μM of resveratrol and at 0.05–10 μM of P11 (Fig. 3). Responders to P4 (n = 8) generated no statistically significant changes in the response to the agent.

Resveratrol, derivatives P4 and P11 were shown to be less toxic in primary human erythroid cultures than in K562 cells with no major reduction in cell survival for concentrations below 20 μM for resveratrol and P4 and for any concentration tested for P11, in both responders and non-responders (Figs. 3 & 4). P1 showed higher cytotoxicity than resveratrol with cell survival dropping to <50% at concentrations of 5 μM and above. P1 was therefore considered inappropriate as a therapeutic HbF inducer. Interestingly, treatment with 0.1 μM concentration of derivative P4 in responders and 0.01 μM of P4 in non-responders, increased cell numbers significantly (p-value < 0.05, paired t-test) above those observed with methanol alone, an effect that was not observed with P11 or resveratrol, suggesting a proliferative role at low concentrations of the agent.

The concurrent application of several HbF inducers is seen as a possible method that would allow synergistic enhancement of their individual activities [40]. Therefore, the combined use of resveratrol with decitabine, a known HbF inducing agent, was investigated as an alternative therapeutic approach as their simultaneous use could combine the antioxidant activity of resveratrol with the strong HbF inducing activity of decitabine. The combined use of decitabine with resveratrol in K562 cells (Fig. 5) increased hemoglobin production significantly above the

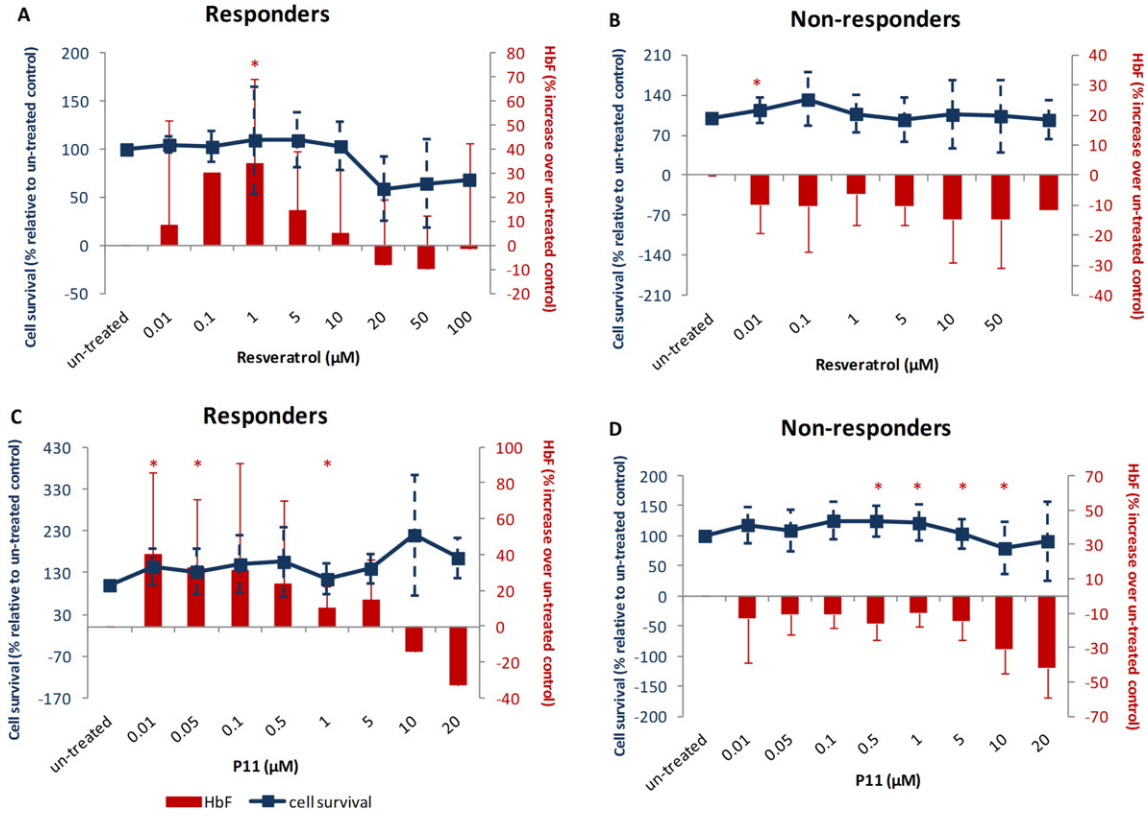

Fig. 3. Treatment of primary human erythroid cultures from healthy donors with Resveratrol (A + B) and P11 (C + D). Primary human erythroid cultures were divided into responders (A + C) and non-responders (B + D) to each agent based on a 10% increase threshold in HbF percentage as determined by HPLC analysis following treatment. The results are the average of 5–7 cultures. Error bars correspond to the standard deviation. Statistically significant changes in HbF levels according to the paired t-test (p-value < 0.05), are marked with *.

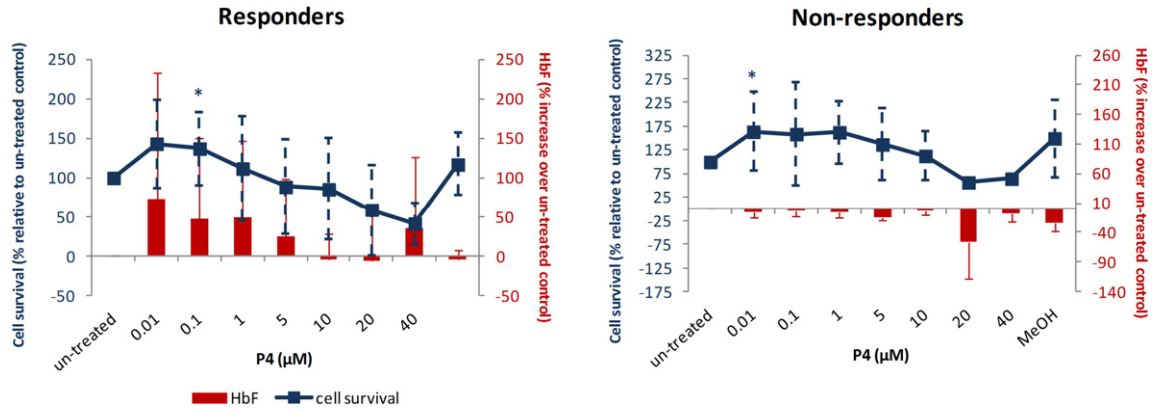

Fig. 4. Treatment of primary human erythroid cultures from healthy donors with P4. Primary erythroid cultures were divided into responders (A) and non-responders (B) based on a 10% increase threshold in HbF percentage as determined by HPLC analysis. The results are the average of 4–8 cultures. Error bars correspond to the standard deviation. Statistically significant changes in cell survival according to the paired t-test (p-value < 0.05) above the vehicle treated cells are marked with *.

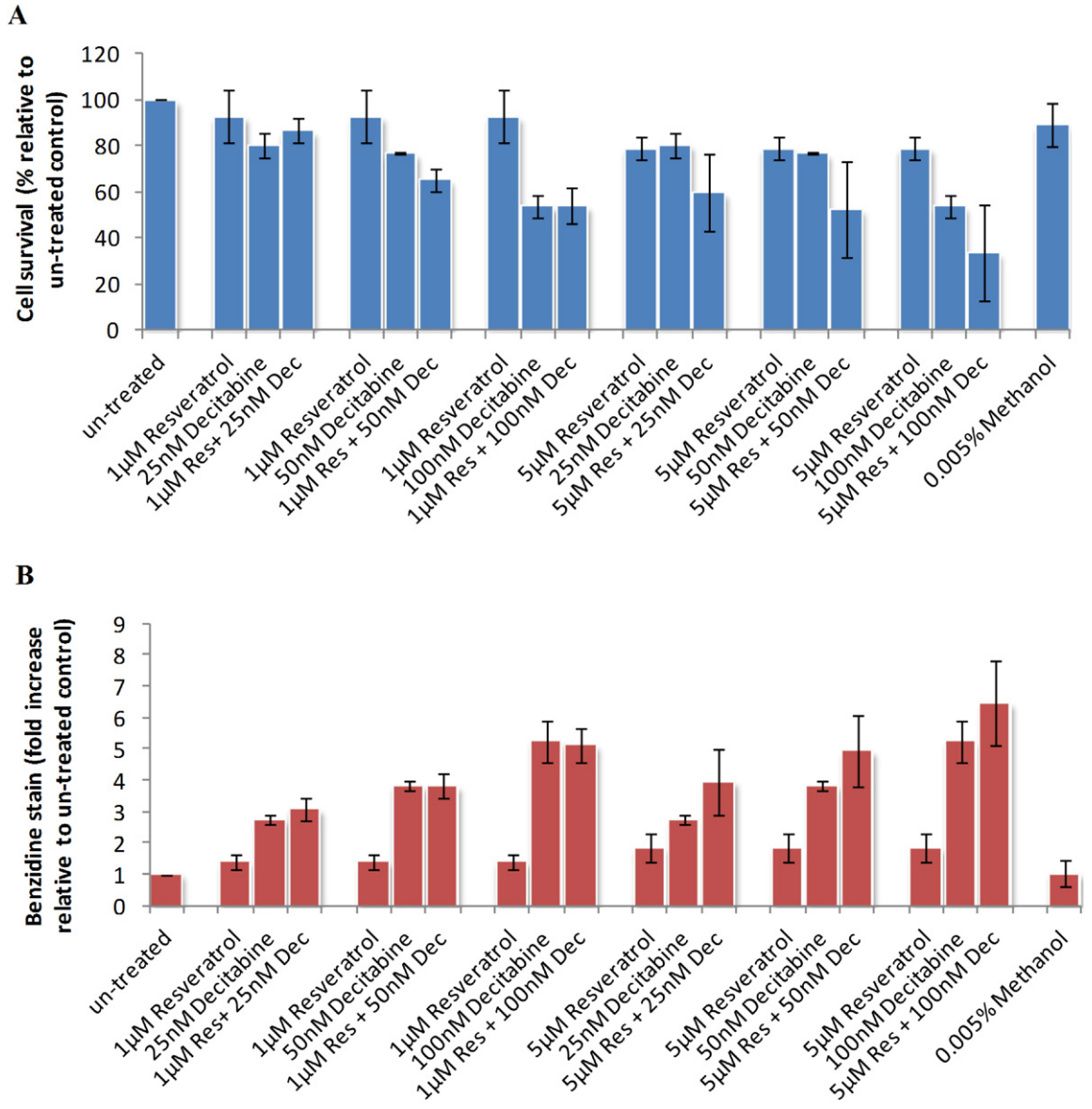

Fig. 5. Dual treatment with resveratrol and decitabine in K562 cells. Cell survival (A) and hemoglobin induction (B) were measured for each agent alone and in combination with the second agent. A significant increase in hemoglobin production (p-value < 0.05, paired t-test) was observed between the combinatorial regime and both concentrations of resveratrol alone, but not significantly above any of the concentrations of decitabine alone. Cytotoxicity was not statistically increased above the additive effect of the agents and was only occasionally significantly (p-value < 0.05) increased above the toxicity of each agent alone. The results represent the average of three experiments with error bars corresponding to the standard deviation.

effect of resveratrol alone (p value < 0.05) for both concentrations of resveratrol tested. Although the increase of hemoglobin production with the combined regime was higher than the effect of decitabine alone, this increase was not statistically significantly (p-value > 0.05) above the effect of decitabine alone. In particular, the strongest response was obtained when 100 nM decitabine was administered with 5 μM resveratrol, resulting in a 6.5 fold increase in the percentage of hemoglobin containing cells, an increase that was significantly higher than the 1.83-fold increase observed with resveratrol alone and non-significantly higher than the 5.2 increase observed with decitabine alone (Fig. 5). However, the combinatorial effect observed was significantly lower than the additive effect of both agents together at all concentrations tested (p-value < 0.05, paired t-test), suggesting competition between the two agents for common down-stream targets or saturation of common targets. Additionally, the cytotoxicity of the combinatorial regime was not significantly different from the additive toxicity of the two agents alone (p-value > 0.05, paired t-test) and only occasionally significantly greater than the cytotoxicity of each agent (p-value < 0.05, paired t-test). Despite the fact that the combined therapy increased hemoglobin production below the additive effect of the agents, the small increase above the effect of each agent alone and the lack of major increase in cytotoxicity, still renders this approach as beneficial.

4. Discussion

A number of studies have led to the identification of different classes of pharmacological agents able to reactivate HbF synthesis both *in vitro* and *in vivo*. For many of these chemicals, however, there are concerns over their toxic and carcinogenic potential in humans. Studies have shown that resveratrol not only exhibits antioxidant activity, but can also stimulate the expression of the γ-globin genes [34]. In the current study, we have shown that resveratrol can increase hemoglobin production in K562 cells. However, increasing concentrations of resveratrol in K562 cells resulted in a dose-dependent reduction in cell survival confirming its anti-proliferative effect, possibly mediated through cell cycle arrest and induction of ER stress as shown by Liu et al. [41]. Due to this anti-proliferative effect, identification of resveratrol derivatives which exhibit potent hemoglobin-inducing properties with lower cytotoxicity is important. In an effort to identify such compounds, nine novel resveratrol derivatives were synthesized and tested in K562 cells. Out of these, only three derivatives (P1, P4 and P11) exhibited hemoglobin inducing activity comparable to the parent compound in K562 cells, with only one (P11) being less cytotoxic. Although previous studies [34] have shown that resveratrol also has an effective HbF inducing activity in primary human erythroid cultures, our results show that none of the agents tested, including resveratrol, have a uniformly potent HbF inducing activity in all the primary human erythroid cultures tested. For each agent tested, some cultures responded quite strongly, in terms of increase in the levels of HbF, while others responded poorly. Therefore, cultures were grouped into responders and non-responders based on their HbF increase following induction with each agent. Resveratrol and derivative P11 showed a statistically significant increase in HbF levels, within the responder group, at 1 and 0.01 μM concentrations, respectively. In contrast, both agents reduced HbF levels below the levels observed in the un-treated control, within the non-responder group. The above findings suggest that the effectiveness of the two agents is limited due to their lack of uniform HbF induction in primary erythroid cultures.

The low HbF induction observed in primary human erythroid cultures of responders does not coincide with the high hemoglobin-inducing activity of resveratrol observed in K562 cells. In the K562 cell line, resveratrol is shown to promote differentiation that might not necessarily correspond to an increase in the percentage of HbF in primary human erythroid cultures. In fact, Franco et al. [42] showed that resveratrol accelerates erythroid cell maturation of CD34+ erythrocytes from healthy and β-thalassaemic patients, through up-regulation of FOXO3a rather than increasing the expression of γ-globin gene expression. It would therefore be interesting to investigate whether the three derivatives have the ability to promote erythroid cell maturation despite the variable effect in HbF production in primary erythroid cultures.

Furthermore, with a more optimal induction protocol the increase in HbF production observed following the addition of test chemicals to primary cultures might be more significant. Lancon et al. [43] demonstrated that cellular uptake of resveratrol is reduced by two fold in medium containing 10% serum compared to serum-free medium. Additionally, this group showed that increasing concentrations of BSA reduced the uptake of resveratrol by two fold at a concentration of 3.5 g/L albumin. The primary human erythroid cells used for our screening experiments were grown in 30% serum and 11.3 g/L BSA in Phase II. We therefore suggest that the effect of each agent should be confirmed in an alternative *ex vivo* experimental model.

Despite the lack of statistically significant HbF induction in primary erythroid cells, P4 significantly increased cell numbers compared to the vehicle-treated cultures, a finding that was not observed for resveratrol and P11. The three derivatives, although similar in structure, demonstrate important functional differences. One of the most easily observable structural differences which could explain some of the biological effects seen is the lack of substituents on the aromatic ring in the case of P4 compared to the presence of ortho, para and meta substituents on resveratrol and the other derivatives.

Therapeutic approaches for the treatment of β-thalassemia should be directed towards improving the clinical picture of the patients. However, most HbF inducers, including 5-azacytidine and butyrate do not have the ability to improve the clinical picture due to their low HbF inducing activity, cytotoxicity and side effects. We therefore moved on to investigate the combined use of resveratrol and decitabine with the potential of increasing the HbF inducing activity of the two agents while combining it with resveratrol's antioxidant activity. Decitabine is a hypomethylating agent that is known to have a chemopreventive rather than carcinogenic effect while increasing HbF levels [44]. In contrast, resveratrol has antioxidant activity, a crucial property in beta-thalassemia, and can inhibit ribonucleotide reductase in a similar manner to hydroxyurea [33]. On preliminary screening on K562 cells we have shown that a combinatorial regime of 5 μM resveratrol and 100 nM decitabine increases the percentage of hemoglobin producing cells by 6.5 fold. The increase in hemoglobin containing cells was greater than the effect observed using each agent alone, but was only statistically significantly elevated above the effect of resveratrol alone. Moreover, the increase observed with the combinatorial regime is lower than the additive effect of the two agents suggesting competition between the two agents for common down-stream targets or saturation of common targets. Despite the minimal synergistic induction in hemoglobin production offered by the combinatorial regime in K562 cells, the combination should be investigated further in primary human erythroid cultures and thalassemic mouse models to study the effect of the combinatorial therapy specifically on HbF levels and the phenotypic picture of the disease, respectively. The use of a combinatorial approach as a method for pharmacological reactivation of HbF is still interesting as can be seen by the increase in HbF levels in CD34+ erythrocytes when treated with pomalidomide and HU [40]. Furthermore, studies regarding the HbF inducing activity of resveratrol are still controversial and thus further investigation of resveratrol and its derivatives as a therapeutic approach of β-thalassemia is indicated.

In conclusion, we have shown that three resveratrol derivatives can increase the percentage of hemoglobin containing cells in K562 cell line with P11 being less cytotoxic than resveratrol. A respective increase in the levels of HbF was replicated only in a small number of primary human erythroid cultures derived from healthy volunteers suggesting a variable response to the agents. Further investigation of resveratrol and its derivatives is encouraged in different experimental models. In addition, the synergistic effect of resveratrol and decitabine on hemoglobin production was only minimal in K562 cells.

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Competing interests statement

No authors of this article have any commercial or financial interest in the products described in this article.

Author contributions

All authors have contributed sufficiently to the project to be included as authors. A.T., M.P., P.S., R.G. and M.K. conceived and designed the experiments, L.F. and M.R.C synthesized the compounds, A.T. performed the experiments, analyzed the data and wrote the manuscript under the guidance of M.P., M.K and S.L.T.

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