Selected terpenes from leaves of *Ocimum basilicum* L. induce hemoglobin accumulation in human K562 cells

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Re-expression of fetal hemoglobin (HbF) was proposed as a possible therapeutic strategy for \(\beta\)-haemoglobinopathies. Although several inducers of HbF were tested in clinical trials, only hydroxyurea (HU) received FDA approval. Despite it produced adequate HbF levels only in half of HU-treated SCD patients, and was ineffective at all in \(\beta\)-thalassemia patients, beneficial effects of this approach suggested to continue in this direction identifying further molecules capable of inducing HbF. We tested the potential of essential oil isolated from *Ocimum basilicum* L. leaves (ObEO) in inducing hemoglobin biosynthesis. Initially, dose-dependent effect and kinetics of hemoglobin accumulation in K562 cells after treatment with ObEO were evaluated. ObEO induced dose-depen-dent hemoglobin accumulation superior to hydroxyurea and rapamycin and a stronger \(\gamma\)-globin mRNA expression. Terpenes composition of ObEO was studied by GC-MS. Three main constituents, linalool, eugenol and eucalyptol, represented about 75% of total. A blend of these three terpenes fully replicated the ObEO's biological effect, thus indicating that one of them or all together could be the active ingredients. When terpenes were tested individually, eugenol was the only one inducing stable hemoglobin accumulation, while eucalyptol and linalool produced only a small transient response. However, eugenol potential was strongly enhanced in the presence of eucalyptol and linalool, suggesting a synergistic effect on hemoglobin accumulation. By these results, the discovery of a new inducer and the interesting activity of a blend of major terpenes from ObEO on HbF accumulation could have positive outcomes on \(\beta\)-thalassemia and sickle cells anemia.

1. Introduction

Hemoglobinopathies such as sickle cell anemia (SCD) and \(\beta\)-thalassemia are hereditary pathologies of the globin chains leading to anomalous hemoglobin (Hb) molecules. In normal subjects, switch-off of \(\gamma\)-globin gene transcription occurring shortly after the birth stops the production of fetal Hb (HbF, \(\alpha_2\beta_2\)) into erythroid precursors and, in the meantime, the increase of \(\gamma\)-globin gene expression permits its functional replacement with adult hemoglobin (HbA, \(\alpha_2\beta_2\)). In pathological conditions, Glu→Val substitution in the \(\beta\)-globin subunit results in sickle Hb (HbS) typical of SCD that provokes the sickling of red blood cells, whereas, in \(\beta\)-thalassemia, the total absence (\(\beta^0\)-thalassemia) or a marked decrease (\(\beta^+\)-thalassemia) of normal \(\beta\)-globin subunits determine excess accumulation of free \(\alpha\)-subunits incapable of forming normal HbA tetramers [1,2]. Natural increment of the HbF level blocks HbS polymerization in SCD improving symptoms, while it permits replacement of HbA function in \(\beta\)-thalassemia [3]. Therefore, molecules enhancing the \(\gamma\)-globin expression, and therefore leading to re-expression of HbF, were proposed as a possible strategy to improve the therapeutic outcome in patients with \(\beta\)-hemoglobinopathies.

Abbreviations: SCD, sickle cell anemia; Hb, hemoglobin; HbF, fetal hemoglobin; HbA, adult hemoglobin; HbS, sickle hemoglobin; HU, hydroxyurea; EO, essential oil; ObEO, *Ocimum basilicum* L. essential oil; GC-MS, gas chromatography–mass spectrometry; mRNA, messenger ribonucleic acid; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; RPMI, Roswell Park Memorial Institute medium; FDA, Food and Drug Administration; NIST, National Institutes of Standards and Technology; EPA, Environmental Protection Agency; NIB, National Institute of Health.

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Several inducers of HbF were tested in clinical trials [4–7], but only hydroxyurea (HU) drug received the FDA approval. Despite adequate HbF levels were observed only in half of the HU-treated SCD patients, and it was ineffective at all in β-thalassemia patients [8], the beneficial effects of this therapeutic approach [9,10] have stimulated the interest in identifying further molecules capable of inducing HbF.

Vegetables were used largely in traditional medicine and represented a natural reservoir for future therapeutic drugs. The genus Ocimum, belonging to the Lamiaceae family, includes 50–150 species [11]. Ocimum basilicum L. (sweet basil) is an annual herb growing worldwide [12]. The essential oil (EO) derived from this plant was used in traditional medicine for different applications, especially in many Asian and African countries [13].

In this work, we studied the effects of EO from Ocimum basilicum L. (ObEO) on Hb accumulation and γ-globin gene expression in human K562 cells. After GC-MS characterization of its compositions, we analysed the principal terpenes responsible of the biological effects and modified the proportion of them to obtain a blend with highest inducing effects as possible on Hb accumulation.

2. Results and discussion

Since human K562 cell line accumulates Hbs if appropriately stimulated, these cells represent a valuable model for the screening of novel molecules that increase the HbF level [14–16]. Herein, it was used to study the time-drive and dose-dependent effects of ObEO on Hb accumulation. Cells cultured in the absence or in the presence of increasing concentrations of ObEO (from 0 to 5 μg/mL) were analysed after 4, 5 and 6 days of incubation. At each time-point, total Hb accumulation was evaluated by staining the cells with benzidine, which recognizes the heme group, colouring the cells in intense blue. HbF levels at the same time point were presumed analysing the γ-globin gene expression by real-time RT-qPCR (Fig. 1A).

As positive control, the cells were treated with 10 nM Rapamycin, an inducer of HbF accumulation that we have previously reported to be more potent than the HU not also in human K562 cells but also in erythroid precursors obtained from β-thalassemia patients [17]. The cells showed a significant accumulation of Hb (p < 0.05) with respect to untreated control cells already after 5 days of treatment with ObEO at the minimum concentration tested. Higher accumulation of Hb was observed 6 days after the administration of ObEO at the maximum concentration (5 μg/mL), with a good inducing effect comparable to that obtained with 10 nM Rapamycin. HbF accumulation was always accompanied by an increase in γ-globin mRNA, suggesting that treatment could rise up the HbF levels into cells.

In addition, at the same time-points tested above, the inducing effects of ObEO on γ-globin mRNA expression were assayed in RT-qPCR using the β-actin mRNA as normalizing reference. The increase or reduction of γ-globin expression after the ObEO administration at day 4, with respect to untreated control cells, was expressed as fold, calculated using the formula: fold = 2^ΔΔCt. Fold values below 0.5 and upper to 2.0 indicated that treatment produced significant modulation of γ-globin gene expression. From the data reported in Fig. 1A, right panel, γ-globin mRNA expression increased over the days of treatment at each dosage tested, with a maximum effect observed at day 6. At the higher concentration used (5 μg/mL), a saturation effect was clearly evident at day 6 of treatment. In general, the observed increase of γ-globin mRNA expression, paralleled well with the accumulation of Hb into the cells. Noteworthy, the observed increase in γ-globin mRNA expression, compared to basal condition present in untreated cells, was >16 times higher after 6 days of treatment with 5 μg/mL ObEO, while it was only 4 times increased using 10 nM rapamycin. As the two treatments determined a comparable percentage of benzidine positive cells (about 24%), we could infer that the ObEO treatment results in higher Hb accumulation into cells than the reference drug.

In order to verify whether Hb accumulation was a general feature of EO or, rather, if it was a specific feature of some one of them, we also analysed the biological activity of other EOs. The EOs from Rosmarinus officinalis, Thymus vulgaris, Cananga odorata and Cymbopogon citratus were administered at highest concentration tested in the experiments described above (5 μg/mL) and benzidine positive cells were evidenced after 5 days of treatment (Fig. 1B). As control of Hb accumulation, cells were treated with 10 nM Rapamycin or 50 μM HU. Among the EOs tested, only ObEO induced Hb accumulation into cells, at levels comparable to those obtained by rapamycin treatment and higher than that obtained with HU. These data suggested that Hb accumulation should be an ObEO peculiarity.

Although EOs are complex mixtures of about 20 to 60 different components, few terpenes and low molecular weight terpenoids present at high concentrations usually determine their biological activity [18]. However, biological effect could be due to the synergy of all terpenes, including those present in small quantities [19]. Therefore, we performed the GC-MS analysis of ObEO aiming to characterize the chemical components responsible for Hb accumulation. Twenty monoterpene was identified and three predominant monoterpene represented about the 75% of the blend (eucalyptol, 7,39 ± 0.28%; linalool, 55,44 ± 2.11%; eugenol, 12,22 ± 0.46%) (Table 1).

To clarify contribute of predominant terpenes to ObEO biological effect, we prepared a blend of pure chemical terpenes (herein termed minimum-EO) mixing linalool, eugenol and eucalyptol at the same concentration found in the ObEO, using dimethyl sulfoxide as solvent. Its dose-dependent and time-dependent effects on total Hb accumulation (Fig. 2A) and γ-globin expression (Fig. 2B) in K562 cells were evaluated, that were compared with those obtained with ObEO treatment. Effects on Hb accumulation were very similar (p < 0.05), suggesting that the biological effect observed was solely due to the main components of the oil.

Following, we analysed by GC-MS the composition of EOs from Rosmarinus officinalis, Cymbopogon citratus, Thymus vulgaris and Cananga odorata that were unable to induce Hb accumulation (see Fig. 1A) that was compared with that of ObEO in order to highlight any chemical differences. The main components of Rosmarinus officinalis’ EO were α-pinene 10,11 ± 0.38%, β-pinene 7,71 ± 0,29%, camphor 13,27 ± 0,51%, eucalyptol 50,63 ± 1,92% (representing the 81,73 ± 3,11%), linalool was present in moderate quantity (0,77 ± 0,03%) while eugenol was absent. The main components of Cymbopogon citratus’ EO were β-pinene 22,47 ± 0,85%, cis-citral 59,19 ± 2,25%, nerol 4,98 ± 0,19% (representing the 86,64 ± 3,29%), linalool and eucalyptol were present in moderate quantity (2,41 ± 0,09% and 0,47 ± 0,02%, respectively) while eugenol was absent. The main components of Thymus vulgaris’ EO were γ-terpinene 13,16 ± 0,50%, thymol 37,78 ± 1,43%, isothymol 23,15 ± 0,88% (74,09 ± 2,81% of the total), linalool and eucalyptol were present in moderate quantity (3,31 ± 0,13% and 1,71 ± 0,06%, respectively) while eugenol was absent. From these data, among the terpenes predominant in ObEO, eugenol was the only one absent in other tested EOs and, therefore, we hypothesized that eugenol could be an active principle of ObEO responsible for Hb accumulation. By contrast, presence of high amounts of eucalyptol in Rosmarinus officinalis’ EO, and linalool in Cananga odorata’s EO, associated with their inability to induce Hb accumulation, suggested that such terpenes should not be influential for triggering the biological effect.

The contribution of single terpenes in Hb accumulation was further explored. K562 cells were cultured in the presence of increasing
amounts of linalool, eucalyptol or eugenol and Hb accumulation analysed 5 days after administration (Fig. 3). The data shown in Fig. 3B evidenced the ability of eugenol to induce dose-dependent Hb accumulation, with a plateau effect reached at 5 μg/mL, whereas eucalyptol and linalool did not produce any significant effect within the range of tested concentrations. Thus eugenol, among the several pharmacological and toxicological activity already described [20], showed the additional ability to stimulate the accumulation of hemoglobin and adds therefore to the limited number of small molecules that can potentially address genetic diseases. Like as hydroxyurea, eugenol has important molecular properties (low molecular weight, adequate partition coefficient, polar surface area, number of hydrogen bond donors and acceptors and others) that allows to hypothesize its possible oral administration. Comparing the effect of single administration of eugenol on Hb accumulation with that of minimum-EQ (in equivalent quantity permitting to obtain the same concentration of eugenol in the culture medium), we observed that minimum-EQ was more potent. For example, 25% of cells exhibited Hb accumulation in the presence of 5 μg/mL minimum-EQ at day 5 (see Fig. 2A, black bar), a solution in which eugenol represents 12.22% corresponding to a final concentration of 0.6 μg/mL eugenol. At this concentration, eugenol induced accumulation only in 3–4% of the cells (Fig. 3B, open circles). Since neither
Table 1
GC-MS analysis of the chemical constituents found in the ObEO. The values are expressed as mean±standard deviation of triplicate assays.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Peak area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Pinene</td>
<td>7.81</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td>2</td>
<td>Camphene</td>
<td>8.52</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>3</td>
<td>β-Pinene</td>
<td>10.12</td>
<td>0.92±0.03</td>
</tr>
<tr>
<td>4</td>
<td>Myrcene</td>
<td>11.42</td>
<td>0.41±0.02</td>
</tr>
<tr>
<td>5</td>
<td>Eucalyptol</td>
<td>14.10</td>
<td>7.39±0.28</td>
</tr>
<tr>
<td>6</td>
<td>β-Trans-ocimene</td>
<td>15.84</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>7</td>
<td>Linalool</td>
<td>21.16</td>
<td>55.44±2.11</td>
</tr>
<tr>
<td>8</td>
<td>Camphor</td>
<td>24.46</td>
<td>0.92±0.03</td>
</tr>
<tr>
<td>9</td>
<td>α-Terpineol</td>
<td>30.37</td>
<td>2.98±0.11</td>
</tr>
<tr>
<td>10</td>
<td>Bornylacetate</td>
<td>41.13</td>
<td>1.41±0.05</td>
</tr>
<tr>
<td>11</td>
<td>Eugenol</td>
<td>50.89</td>
<td>12.22±0.46</td>
</tr>
<tr>
<td>12</td>
<td>β-Elemene</td>
<td>53.34</td>
<td>2.11±0.08</td>
</tr>
<tr>
<td>13</td>
<td>α-Cadinenol</td>
<td>57.90</td>
<td>3.49±0.13</td>
</tr>
</tbody>
</table>

llinalool nor eucalyptol induced Hb accumulation up to 10μg/mL (Fig. 3B, filled circles and filled triangles, respectively), we suggested that linalool and/or eucalyptol presence could have enhanced the inducing effect of eugenol.

To test this hypothesis, we mixed the three terpenes in different combinations and verified the Hb accumulation after 5 days of treatment (Fig. 3C). The combination of 2.5μg/mL eucalyptol or linalool with 2.5μg/mL eugenol did not produce higher Hb accumulation than observed following treatment with single terpenes (5μg/mL), thus excluding a direct synergic effect of linalool or eucalyptol with eugenol. Conversely, combination of all three terpenes at the 2.5μg/mL concentration produced higher Hb accumulation than that observed after treatment with single terpenes at the concentration of 7.5μg/mL, suggesting that concomitant presence of linalool and eucalyptol in the mixture synergistically boosted the inducing effect of eugenol. In this respect, it has been described that eucalyptol and linalool modify membrane permeability [21,22], the last increasing the influx of doxorubicin [22]. As a possible rationale for the observed synergy, we speculate that the concomitant administration of linalool and eucalyptol with eugenol may possibly increase the eugenol influx through the membrane, thus increasing its inducing effect on hemoglobin accumulation.

3. Conclusions

Our data demonstrated that ObEO, among the other biological effects till now described, is a powerful inducer of Hb accumulation and γ-globin expression. The terpene responsible for this effect was eugenol, whose activity was never been described before in literature, even if the additional presence of eucalyptol and linalool synergistically enhances its effect. In view of these results, the optimized mixture of these three terpenes that we have described could have beneficial effects in the treatment of SCD and β-thalassemia.

4. Materials and methods

4.1. Materials

Commercial ObEO from leaves, linalool chemotype, origin Egypt (density = 0.928 g/mL), was obtained from a local grocery. Cananga odorata EO (density = 0.964 g/mL) was from CTM, Verona, Italy. Cymbopogon citratus EO (density = 0.876 g/mL) was from Fundacion Chankup, Macas, Ecuador. Thymus vulgaris EO, thymol chemo-type (density = 0.895 g/mL), was from Extrasyntese, Genay, France. Rosmarinus officinalis EO cineol chemotype (density = 0.932 g/mL) was from Vitalis Dr. Joseph S.r.l., Brunico (BZ), Italy. The EOs were kindly provided by Prof. Sacchetti Gianni (University of Ferrara, Italy). Stock
solutions were stored in the dark at \(-80^\circ \text{C}\) in a glass vial sealed with Teflon cap until their use. Rapamycin, linalool, eucalyptol and eugenol (Sigma-Aldrich, Milan, Italy) were aliquoted and stored at \(-80^\circ \text{C}\) in the dark in a glass vial sealed with Teflon cap until their use.

4.2. GC-MS analysis

The GC-MS was performed essentially as described previously [23]. Briefly, the EOs were analysed using an Agilent 5973 Network quadrupole mass selective detector coupled with an Agilent GC 6850 Series II Network Trace gas chromatograph. A HP-5MS capillary column containing 5%-phenyl-methylpolysiloxane (30 m \times 0.25 mm, film thickness, 0.25\(\mu\)m) was employed. GC operating conditions were as follows: carrier gas, helium with a flow rate of 2\(\mu\)L/min; column temperature program was from 45\(^\circ\)C to 100\(^\circ\)C at 1\(^\circ\)/min, then from 100\(^\circ\)C to 250\(^\circ\)C at 5\(^\circ\)/min; injector inlet temperature, 280\(^\circ\)C; volume injected, 1\(\mu\)L of the EO in dichloromethane; split ratio, 1:40. MS operating parameters were as follows: ionization potential, 70\(\text{eV}\); ion source temperature, 230\(^\circ\)C; quadrupole temperature 150\(^\circ\)C, solvent delay 4.20\(\text{min}\), mass range 35–300\(m/z\). The GC retention and mass spectra of peaks obtained were compared with those of authentic standards from NIST/EPA/NIH database and mass spectra from the literature [24,25].

4.3. Cell culture conditions

The human K562 cells were cultured in a humidified atmosphere of 5% \(\text{CO}_2\)/air in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% foetal bovine serum. Cell growth was determined by Burker’s chamber. ObEO was administered to the cells at the seeding day only.
4.4. Determination of Hb accumulation in cells by benzidine stain

The cells were suspended in a solution containing 0.2% benzidine in 5M glacial acetic acid, 10% H2O2, as elsewhere described [17]. Briefly, benzidine positive cells were counted by optical microscope. At least four optical fields containing 50–100 cells were observed and cells stained in intense blue were expressed as percent with respect to total cells.

4.5. RNA isolation

RNA isolation was carried out by using guanidine isothiocyanate (TRIzol reagent, Invitrogen Corporation, Carlsbad, CA). Cells were lysed in 1 mL of TRIzol reagent and 200 μL of chloroform was added. The mixture was vigorously shaken, incubated at room temperature for 10–15 min, and centrifuged at 12,000 xg for 15 min. The aqueous phase was collected and the RNA was precipitated by isopropanol addition. The pellet obtained was washed in 75% ethanol and dissolved in 10 mM Tris-HCl pH 7.5, 1 mM EDTA.

4.6. Real-time RT-qPCR analysis

RNAs (2 μg) were reverse transcribed (RT) with the iScript™ cDNA Synthesis Kit (Bio-Rad), using iScript™ cDNA Synthesis Kit (Bio-Rad) and the cDNA was used as template in gene-specific amplification performed on the StepOnePlus Real-Time PCR System using the StepOne software v.2.3 (Thermo Fisher Scientific). The primers for γ-globin and β-actin mRNAs were described elsewhere [14]. Reactions were performed in a 50 μL volume containing 25 μL SYBR green PCR master mix (Thermo Fisher Scientific) containing the ROX internal passive reference dye, 0, 5 μM of each primer, optimized MgCl₂ concentration between 1.5–3 mM. All determinations were performed in triplicate wells. Endpoint amplified products were subjected to melt curve analysis. Relative quantity of target transcript in the sample was calculate with respect to the reference β-actin mRNA using a comparative CT (∆ΔCt) method. The relative value was expressed as 2–ΔΔCt.

Conflicts of interest

Authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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