Discovery of Eucalyptin C, derived from the fruits of *Eucalyptus globulus* Labill., as a novel selective PI3Kγ inhibitor for immunosuppressive treatment

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[ABSTRACT] The fruits of *Eucalyptus globulus* Labill. are known to have a plenty of medicinal properties, such as anti-tumor, anti-inflammatory, and immunosuppressive activity. Our previous study found that the phloroglucinol-sesquiterpene adducts in the fruits of *E. globulus* were immunosuppressive active constituents, especially Eucalyptin C (EuC). Phosphoinositide 3-kinases-γ (PI3Kγ) plays a pivotal role in T cell mediated excessive immune responses. In this study, EuC was first discovered to be a novel selective PI3Kγ inhibitor with an IC₅₀ value of 0.9 μmol·L⁻¹ and selectivity over 40-fold towards the other PI3K isoforms. Molecular docking, molecular dynamics simulation, and cellular thermal shift assay showed that EuC bound to PI3Kγ. Furthermore, EuC suppressed the downstream of PI3Kγ to induce the apoptosis and inhibit the activation of primary spleen cells derived from allergic contact dermatitis mice. This work highlights the role of the fruits of *E. globulus* as a source of bioactive plant with immunosuppressive activity.

[KEY WORDS] Phosphoinositide 3-kinase-γ (PI3Kγ); Eucalyptin C; Immunosuppressive effects; Activated spleen cells; Phloroglucinol-sesquiterpene adducts

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Introduction

*Eucalyptus globulus* Labill., a tall timber tree, mainly grows in Guangxi and Yunnan Provinces of Mainland China. The fruits of *E. globulus*, or “Yi-Kou-Zhong” in Chinese, are known to exhibit a plenty of medicinal properties, such as anti-tumor, anti-inflammatory, anti-fungal, and anti-dermatitis effects. Dermatitis is related to abnormal enhancement of the immune system. Our previous study reported that the extracts of the fruits of *E. globulus* exerted immunosuppressive effects and contained several phloroglucinol-sesquiterpene adducts, especially eucalyptin C (EuC), one of the potential biological constituents [1]. Further studies demonstrated that EuC was an immunosuppressive agent with high effectiveness and low toxicity [1]. However, the biological targets and mechanisms of action of EuC are still unclear.

Phosphoinositide 3-kinases (PI3Ks) are the key components of the PI3K/AKT pathway associated with various cellu-
lolar activities \[^2\,^3\]. According to their structure preference and substrate specificity, PI3Ks can be categorized into three classes (I, II, and III). Class I PI3Ks are divided into two subfamilies (IA and IB) depending on the receptors to which they couple \[^4\,^5\]. Among these isoforms, PI3K\(\gamma\) (the only isoform of class IB), is over-expressed in immune cells with limited expression in normal cells, and plays a pivotal role in T cell mediated excessive immune responses \[^6\,^7\]. Agents targeting PI3K\(\gamma\) can selectively inhibit the viability of T cells, rather than other cells, which makes it probably a promising target for immunosuppressants with high effectiveness and low toxicity \[^8\].

In the present study, EuC was discovered as a potential PI3K\(\gamma\) inhibitor for the first time and also demonstrated to directly bind to PI3K\(\gamma\). Furthermore, EuC significantly induced the apoptosis and inhibited the differentiation of primary spleen cells derived from allergic contact dermatitis (ACD) mice through the PI3K\(\gamma\) pathway. These data preliminarily suggested that the anti-dermatitis activity of the fruits of *E. globulus* were related to its immunosuppressive effects, and the phloroglucinol-sesquiterpene adducts might act as potential active constituents.

**Materials and Methods**

**Materials**

Eucalyptin C (purity > 98\%) and other test compounds were isolated from the fruits of *E. globulus* in our laboratory \[^1\]. Fetal bovine serum and RPMI 1640 medium were purchased from Invitrogen (San Diego, CA, USA). Cell counting kit-8 was purchased from Dojindo Laboratories (Tokyo, Japan). LDH release kit, Hoechst 33258 staining kit, and Annexin V-PI double staining kit were obtained from KeyGEN BioTECH (Nanjing, China). BCA protein determination kit, RIPA lysis, and Western-blots sample buffer were purchased from Byototime (Shanghai, China). ADP-Glo Max assay kit was purchased from Promega (WI, USA). All human PI3K isoforms were purchased from Millipore (Billerica, MA). FITC was purchased from MedChemExpress (New Jersey, USA). Mouse ELISA kits for IL-4, IFN-\(\gamma\), IL-6, IL-10, and TNF-\(\alpha\) were purchased from Abcam (MA, USA). Primary rabbit antibodies to Bax, Bcl-2, caspase-3, caspase-9, cytochrome c, and PI3K\(\gamma\), were purchased from Cell Signaling Technology (MA, USA). Primary rabbit antibodies to p-AKT, AKT, p-mTOR, mTOR, \(\beta\)-actin, Lamin B, NF-xb, p-IKK\(\alpha\), t-IKK\(\alpha\), p-IKK\(\beta\), IKK\(\beta\), p-I\(\kappa\)B, t-I\(\kappa\)B, and secondary goat-rabbit IgG antibody were purchased from Santa Cruz Biotechnology (CA, USA). All remaining chemicals and reagents used in this experiment were of analytic grade.

**Molecular docking**

Molecular docking was performed using the CDOCKER protocol in Discovery Studio 3.0. The 3D structure of EuC was generated and minimized. The protein was optimized by hydrogenation, dehydration and CHARMM force field. PI3K-\(\gamma\) protein (PDB code: 3L54) was defined as the receptor, and the site sphere was selected on the LXX site \[^9\]. Then, the ligand was removed, while the molecule EuC was placed in the molecular docking process. Maestro software was used for visualization.

**PI3K kinase activity assay**

The enzymatic activity of the kinases was determined by a Promega ADP-Glo Max assay kit. Sample of the kinases (20 mmol·L\(^{-1}\) PI3K-\(\alpha\), PI3K-\(\beta\), PI3K-\(\gamma\), and PI3K-\(\delta\)) were incubated with different concentrations (0.1–100 μmol·L\(^{-1}\)) of compounds 1–18 for 15 min at room temperature in reaction buffer (15 mmol·L\(^{-1}\) HEPES pH 7.4, 20 mmol·L\(^{-1}\) NaCl, 1 mmol·L\(^{-1}\) EGTA, 0.02% Tween 20, 10 mmol·L\(^{-1}\) MgCl\(_2\), and 0.2 mg·mL\(^{-1}\) bovine-\(\gamma\)-globulins) before addition of ATP/diC\(_{2}\)-PP\(_{2}\) mixture to give final concentrations of 3 mmol·L\(^{-1}\) ATP and 500 μmol·L\(^{-1}\) diC\(_{2}\)P\(_{2}\) substrate (for Class I PI3Ks) or 500 μmol·L\(^{-1}\) diC\(_{8}\)P\(_{2}\) substrate (for Class II PI3Ks). The reaction mixtures were incubated at room temperature for 2 h, to which 25 μL of Promega kit stop solution was added. After 40-min incubation at room temperature, 50 μL of Promega detection mix was added followed by incubation at room temperature for 1 h. Plates were then read on an Envision plate reader in luminescence mode. Data were converted to % inhibition and then plotted as % inhibition vs compound concentration. GraphPad Prism 8.0 software was used to determine IC\(_{50}\) values.

**Molecular simulation study**

MD simulations (100 ns) were performed on PI3K-\(\gamma\) without or with EuC at 300 K at the molecular mechanics level using GROMOS96 43a1 force field in GROMACS. The PI3K-\(\gamma\) - EuC complex was soaked in a cubic box of 0.9% NaCl, and the distance between the solute and the box was 5 Å. Both the systems were minimized using 1500 steps of steepest descent for energy minimization. The resulting trajectories were analyzed using *rms* and *rmsf* utilities of GROMACS. The GROMACS 5.1.2 program was used for MD, and all graphs were prepared using GraphPadP 8.0 software.

**Animals**

Male BALB/c mice (aged 6–8 weeks and weighing 20–22 g) were purchased from Comparative Medicine Centre (Yangzhou University, China). The mice were acclimatized for seven days before use. All animals were maintained under standard housing environment at a temperature 22–25 °C on a 12-h light–dark cycle and allowed free access to food and water. All experimental procedures were approved by the Ethical Committee of China Pharmaceutical University (No. SYXK2016-0011).

**Cellular thermal shift assay (CETSA) and isothermal dose-response fingerprint (ITDRF)**

Cellular thermal shift assay (CETSA) and isothermal dose-response fingerprint (ITDRF) experiments were conduc-
evenly mounted onto slides, and dried. Then, the cells were cold PBS twice and resuspended in 20 μL of DEPC water, at 4 °C for 20 min. The cells were then washed with 3 acetate = fuged, and fixed in Carnoy’s solution (methanol 20 μmol·L−1) for 24 h. Then, the cells were collected, centrifugation at 1 × 10^6 cells/mL and then treated with EuC (1, 10, and 20 μmol·L−1) for 24 h. Fixation of cells were the same as CETSA, where the cells were incubated with EuC at different concentrations at designated temperature.

Establishment of an allergic contact dermatitis (ACD) model of mice

Amouse model of ACD was established according to previous study. Briefly, BALB/c mice were treated with 1.5% FITC solution (in 20 μL acetone and dibutylphthalate (1 : 1, %) on the right ears elicited by 0.6% ACD solution (in 20 μL acetone and dibutylphthalate (1 : 1, % [12]. Briefly, BALB/c mice were treated with 1.5% FITC solution. Acetone and dibutylphthalate was applied as vehicle controls.

Preparation of primary splenocytes from ACD mice

The assay was performed as previously described. Briefly, ACD mice were sacrificed and the spleens were aseptically collected, which were then grounded by crossing through a sterile plastic strainer with 200 mesh. After centrifugation at 1000 g for 5 min, erythrocytes were lysed in red blood cell lysis buffer. Then, cell pellets were washed twice with culture medium. The cell viability (95%) was detected by the trypan blue exclusion method.

Cell viability assay and LDH release assay

Primary splenocytes were seeded into a 96-well at a density of 1 × 10^5 cells/mL and then treated with EuC (1, 10, and 20 μmol·L−1) for 24 h. Then, 100 μL of the supernatant of all wells was collected for LDH detection according to the manufacturer’s instructions. Meanwhile, 10 μL of CCK-8 was added to all the wells before incubation for 3 h. Finally, the OD values at 450 nm was recorded by a microplate reader (Tecan, Austria).

Western blot analysis

Primary splenocytes were seeded into a 10 cm cell-culture dish at a density of 1 × 10^5 cells/mL and then treated with EuC (1, 10, and 20 μmol·L−1) for 24 h or EuC (20 μmol·L−1) for 0.5 h, 1 h, and 2 h. Then, the cells were collected and washed twice with cold PBS. Protein extraction and Western blot were conducted according to our previous study [1]. Blots were probed with primary antibodies to p-AKT, t-AKT, p-mTOR, t-mTOR, p-IKKβ, t-IKKβ, p-p65, p-IκBα (1 : 800 dilution); Bax, Bcl-2, cleaved-caspase-3, cleaved-caspase-9 (1 : 1000 dilution); and cytochrome c, NF-κB, β-actin, and Lamin B (1 : 1000 dilution). Quantitative analysis for original bands were calculated using Image J software.

Statistics

The significances of intergroup differences were determined using one-way analysis. Data are expressed as the means ± SD of the indicated numbers of independent experiments. Statistical significance was determined for P < 0.05.

Results and Discussion

Inhibitory effects of the chlorogluconol-sesquiterpene adducts on P13Kγ

The inhibitory effects of EuC (1) and the other 17
phloroglucinol-sesquiterpene adducts (2–18) isolated from the fruits of *E. globulus* in our previous study (Fig. 1) on PI3Kγ were evaluated. Results showed that compounds 1–8 showed potent PI3Kγ inhibitory effects with IC$_{50}$ values ranging from 0.9 to 10.8 μmol·L$^{-1}$, especially EuC (IC$_{50}$ = 0.9 μmol·L$^{-1}$), while compounds 9–13 showed moderate PI3Kγ inhibitory effects with IC$_{50}$ values between 10 and 20 μmol·L$^{-1}$ (Table 1). The PI3Kγ inhibitory effects of these compounds were corresponding to the structure-activity relationship (SAR) of their immunosuppressive effects in our

**Fig. 1** Chemical structures of compounds 1–18
Table 1  Effects of 1−18 on the activities and selectivity of PI3K isoforms

<table>
<thead>
<tr>
<th>Comp.</th>
<th>P13Kγ IC50 (μmol·L−1)</th>
<th>P13Kα/β IC50 (μmol·L−1)</th>
<th>P13Kδ IC50 (μmol·L−1)</th>
<th>Docking score (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9 ± 0.2</td>
<td>42.6</td>
<td>33.5</td>
<td>54.3</td>
</tr>
<tr>
<td>2</td>
<td>1.0 ± 0.1</td>
<td>41.8</td>
<td>36.3</td>
<td>55.1</td>
</tr>
<tr>
<td>3</td>
<td>7.4 ± 1.2</td>
<td>5.4</td>
<td>2.1</td>
<td>3.2</td>
</tr>
<tr>
<td>4</td>
<td>3.8 ± 0.4</td>
<td>5.1</td>
<td>2.7</td>
<td>5.3</td>
</tr>
<tr>
<td>5</td>
<td>7.2 ± 1.1</td>
<td>2.9</td>
<td>4.4</td>
<td>4.3</td>
</tr>
<tr>
<td>6</td>
<td>1.5 ± 0.3</td>
<td>5.0</td>
<td>2.4</td>
<td>1.9</td>
</tr>
<tr>
<td>7</td>
<td>1.7 ± 0.2</td>
<td>3.2</td>
<td>5.9</td>
<td>2.1</td>
</tr>
<tr>
<td>8</td>
<td>1.8 ± 0.5</td>
<td>4.7</td>
<td>2.8</td>
<td>2.6</td>
</tr>
<tr>
<td>9</td>
<td>5.2 ± 1.2</td>
<td>4.9</td>
<td>2.4</td>
<td>4.2</td>
</tr>
<tr>
<td>10</td>
<td>18.4 ± 3.2</td>
<td>5.4</td>
<td>1.7</td>
<td>2.4</td>
</tr>
<tr>
<td>11</td>
<td>11.7 ± 2.1</td>
<td>1.5</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>12</td>
<td>11.9 ± 2.9</td>
<td>1.9</td>
<td>2.1</td>
<td>3.4</td>
</tr>
<tr>
<td>13</td>
<td>12.4 ± 3.6</td>
<td>1.4</td>
<td>1.2</td>
<td>3.1</td>
</tr>
<tr>
<td>14</td>
<td>21.5 ± 2.5</td>
<td>4.1</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>15</td>
<td>20.6 ± 2.1</td>
<td>46.4</td>
<td>32.9</td>
<td>43.2</td>
</tr>
<tr>
<td>16</td>
<td>54.6 ± 4.4</td>
<td>41.3</td>
<td>35.3</td>
<td>49.9</td>
</tr>
<tr>
<td>17</td>
<td>43.9 ± 5.8</td>
<td>0.5</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>18</td>
<td>52.8 ± 3.9</td>
<td>0.2</td>
<td>1.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* It means the selectivity index and is given as the ratio of IC50 (P13K-γ)/IC50 (P13K-α), IC50 (P13K-β)/IC50 (P13K-γ) or IC50 (P13K-δ)/IC50 (P13K-γ). Data are the mean ± SE in duplicate experiments. 

Previously, compounds of phloroglucinol-monocyclic monoterpene fused pyranoid ring (1 and 2) showed the most potent PI3Kγ inhibitory effects (IC50 < 1 μmol·L−1). In the compounds of the phloroglucinol-sesquiterpene adducts, C-3 position substituted by isovaleryl group (5, 6 and 7, IC50 values ranging from 4.2 to 5.6 μmol·L−1) showed stronger PI3Kγ inhibitory effects than that substituted by aldehyde group (8−16, IC50 values ranging from 10.8 to 54.6 μmol·L−1), indicating that isovaleryl group made contribution to the activity. Furthermore, the PI3Kγ inhibitory effects of these compounds can be well explained by their docking results that compounds with strong inhibitory effects (1−8) possessed low binding energies (ranging from −42.5 to −12.4 kcal·mol−1), while those with poor inhibitory effects (16−18) possessed high binding energies (ranging from −4.3 to −2.1 kcal·mol−1). Compounds with potent PI3Kγ inhibitory effects also exhibited strong immunosuppressive activities, indicating that these compounds exerted immunosuppressive activities mainly through PI3Kγ inhibition.

**Binding mode of EuC towards PI3Kγ**

Molecular docking results showed that EuC (Fig. 2A) well bound to PI3Kγ with a potent -CDOCKER score of 42.5 kcal·mol−1. Further protein-ligand complex analysis showed that EuC interacted with the residues (Glu880, Val882, Tyr867, and Asp964) of PI3Kγ via H-bond (Figs. 2B and 2C). The crystal structure of PI3Kγ suggested that Tyr867 and Val882 of PI3K-γ were mainly responsible for the known inhibitors of PI3Kγ, and these interactions stabilized the switching of the activation loop and positions the substrate in the binding cavity. In addition, the surface representations also suggested that EuC occupied the internal cavity (Fig. 2B). These results indicated that EuC might be a potential PI3Kγ inhibitor.

**Isoform selectivity of the phloroglucinol-sesquiterpene adducts**

As mentioned earlier, PI3Ks can be divided into four isoforms, including PI3Kα, β, δ, and γ. PI3Kγ is specifically expressed in immune cells and plays a key role in T cell-mediated diseases. Therefore, it is necessary to investigate the inhibitory effects of these phloroglucinol-sesquiterpene adducts on the other three isoforms of PI3Ks. As shown in Table 1, only 1, 2, 15, and 16 exhibited over 40-fold selectivity for PI3Kγ compared with the other PI3K isoforms, while other compounds exhibited poor selectivity (< 6-fold). Structural analysis of the phloroglucinol-sesquiterpene adducts is needed to understand the interactions between these compounds and the PI3K isoforms.
ture-selectivity relationship analysis suggested that 1, 2, 15 and 16 bearing cyclization oxygen ring moiety showed better selectivity than the other phloroglucinol derivatives, indicating that the relative rigid structure is more matching lead for their selectivity towards PI3Kγ. Taken together, EuC (1) with the most potent activity and high selectivity, was selected as the lead compound for further studies.

**Structural deviations and compactness**

The best conformation of PI3Kγ-EuC was used as the star conformation for the molecular dynamics (MD) simulation by YASARA. Binding of a small molecule in the binding pocket of a protein can lead to large conformational changes [13]. Root mean square deviation (RMSD) is one of the most important fundamental properties to evaluate the structural stability of a protein [13, 14]. Fig. 3A showed that the average RMSD values for PI3Kγ and PI3Kγ-EuC were found to be 0.07 and 0.09 nm, respectively. The RMSD data suggested that the binding of EuC stabilized PI3Kγ and led to less structural deviations from its native conformation. To further assess local structural flexibility, the average fluctuation of all residues and the root-mean square fluctuations (RMSF) of PI3Kγ on ligand binding were plotted during the simulation as a function of residue number (Fig. 3B). The RMSF data revealed several residual fluctuations in PI3Kγ at several regions of protein structure. These residual fluctuations were minimized upon binding of EuC throughout the simulations in a region spanning from the N-terminal to the C-terminal. These results indicated that EuC may directly bind to PI3Kγ.

**EuC directly binds to PI3Kγ**

To evaluate the binding between EuC and PI3Kγ, thermodynamic experiment was carried out. The cellular thermal

**CETSA and ITDRF were used to evaluate the binding between EuC and PI3Kγ. (A–B) The original bands of CETSA and their quantitative analysis. (C–D) The original bands of ITDRF and their quantitative analysis**
shift assay (CETSA) was used to investigate the thermal stabilization of the proteins upon ligand binding, since this assay has been extensively used to detect the interactions between donors and ligands [15]. The results showed that the content of PI3Kγ in the DMSO group gradually decreased with increasing temperature, while the addition of EuC could effectively inhibit the decrease of PI3Kγ. As shown in Figs. 4A and 4B, after EuC incubated with PI3Kγ, the thermal stabilization of PI3Kγ was enhanced compared with the control group (DMSO), and such thermal stabilization between EuC and PI3Kγ was dose-dependent from IT-DRF cetsa (Figs. 4C and 4D). These results suggested that EuC directly bound to PI3Kγ.

EuC inhibits the differentiation of spleen cells derived from allergic contact dermatitis (ACD) mice to T cells through the PI3Kγ/NF-κb pathway

Allergic contact dermatitis (ACD) is primarily regulated by T cells within the adaptive immune system, where the main pathogenesis is T cell activation [2, 3]. It is reported that PI3Kγ is involved in T-cell receptor (TCR) signaling to induce the nuclear translocation of NF-κb, which is responsible for T cell activation [10]. Therefore, the effects of EuC on T cell activation and the NF-κb signaling pathway were detected to further determine the immunosuppressive effect of EuC on PI3Kγ. In this work, fluorescein isothiocyanate (FITC)-induced ACD mice (Fig. 5A) were used to activate T cells in the spleen and evaluate the inhibitory effect of EuC on T cell activation. Results showed that FITC significantly increased the volume of the ear (Supplementary Fig. S1A) and spleen (Supplementary Fig. S1B), indicating that dermatitis and spleen differentiation occurred. Notably, FITC-induced ACD model directly or indirectly targets on the NF-κb pathway, followed by secretion of cytokines, such as IL-4, IFN-γ, IL-6, and IL-10 of T-helper cells, leading to the development of immune responses in the skin [17-19]. Figs. 5B−5E showed that FITC significantly increased the levels of IL-4, IFN-γ, IL-6, and IL-10 in the spleen of ACD mice, suggesting that spleen cells were activated to differentiate T cells.

Fig. 5 Effects of EuC on the differentiation of active primary spleen cells. (A) Scheme of the experimental protocol for establishment of a mouse model of ACD. (B−E) The levels of IL-4, IL-6, IL-10, and IFN-γ in primary spleen cells derived from ACD mice.

***P < 0.001 vs the control group, and *P < 0.05, **P < 0.01, or ***P < 0.001 vs the ACD mice group (n = 6)
However, treatment with EuC (1, 10, and 20 μmol·L⁻¹) significantly decreased the levels of these cytokines in a dose-dependent manner. Western blot results showed that EuC significantly decreased the phosphorylation of IKKα (Fig. 6A), IKKβ (Fig. 6B), and IκBα (Fig. 6C) in a time-dependent manner. EuC also inhibited the nuclear translocation of NF-κb from the cytoplasm to nucleus (Fig. 6D). In addition, the levels of p65 subunit of NF-κb were also assessed by immunofluorescence assay. Nuclear localization of p65 subunit of NF-κb was determined by immunofluorescence assay. These results suggested that EuC inhibited the nuclear translocation of NF-κb. (E) The subcellular location of the p65 subunit of NF-κb was determined by immunofluorescence assay. *P < 0.05, **P < 0.01, or ***P < 0.001 vs the control group (n = 3).

**Fig. 6** Effects of EuC on the regulation of the NF-κb pathway. (A) The original bands and quantitative analysis of p-IKKα. (B) The original bands and quantitative analysis of p-IKKβ. (C) The original bands and quantitative analysis of p-IκBα. (D) The original bands and quantitative analysis of the intranuclear and extranuclear NF-κb. (E) The subcellular location of the p65 subunit of NF-κb was determined by immunofluorescence assay. *P < 0.05, **P < 0.01, or ***P < 0.001 vs the control group (n = 3).

Evidence has shown that PI3Kγ also participates in the survival of T cells through the PI3Kγ/AKT/mTOR/mitochondrial apoptotic pathway. EuC induces the apoptosis of T lymphocytes through the PI3Kγ/AKT/mTOR/mitochondrial apoptotic pathway. EuC, as a potential PI3Kγ inhibitor, can theoretically inhibit the survival of T cells derived from ACD mice.

Results showed that EuC significantly decreased cell viability (Fig. 7A) and increased LDH release (Fig. 7B) of T cells. Hoechst 33258 staining showed that EuC increased fluorescence intensity of the cells in a dose-dependent manner, indicating that EuC induced the apoptosis of T cells (Fig. 7C). Annexin V-PI double staining further demonstrated that EuC treatment significantly increased the late apoptosis (Fig. 7D). Furthermore, mitochondrial membrane potential (ΔΨm) was measured by determining the red/green fluorescence ratio of JC-1. As shown in Fig. 7E, treatment with different concentrations of EuC (1, 10, and 20 μmol·L⁻¹) resulted in significant ΔΨm loss compared with the control group. These results suggested that EuC induced the apoptosis of T lymphocytes through activating mitochondrial apoptosis. In addition, EuC showed weak cytotoxicity on several normal cells, such as human hepatic WRL-68 cells, human retinal ARPE-19 cells, and human colonic epithelium FHC cells (Supplementary Fig. S2), indicating that EuC might be a T cell selective immunosuppressive agent.
Analysis showed that 20 μmol·L⁻¹ of EuC time-dependently decreased the expression of p-AKT (Figs. 8A and 8B) and p-mTOR (Figs. 8A and 8C). In addition, EuC increased the ratio of Bax/Bcl-2 (Figs. 8D and 8E), inhibited the cytochrome c release from the mitochondria (Figs. 8D and 8H), and decreased the expression of cleaved-caspase-3 (Figs. 8D and}

Fig. 7  Effects of EuC on the apoptosis of active primary spleen cells. (A) CCK-8 assay for cell viability. (B) LDH release in cell supernatant. (C) Hoechst staining for nucleus chromatin condensation. (D) Annexin V-PI double staining, distribution of viable (lower left), necrotic (upper left), late apoptotic (upper right), and early apoptotic (lower right). (E) JC-1 staining for the evaluation of MMP (the intensity of red fluorescence is positively correlated with MMP). *P < 0.05, **P < 0.01, or ***P < 0.001 vs the control group (n = 3)
cleaved-caspase-7 (Figs. 8D and 8F), cleaved-caspase-9 (Figs. 8D and 8G), and PPAR, indicating that EuC can activate the mitochondrial apoptotic pathway. However, a PI3K/AKT inhibitor, ON-01910, significantly weakened the injury effects (Fig. 9A) and mitochondrial apoptotic pathway activation of EuC on T cells (Figs. 9B−9D). These results fully demonstrated that EuC exhibited inhibitory effects against T cells through the PI3K/AKT/mTOR/mitochondrial apoptotic pathway.

Conclusions

In summary, the phloroglucinol-sesquiterpene adducts in the fruit of *E. globulus*, are first demonstrated to be new PI3Kγ inhibitors, especially EuC with an IC₅₀ of 0.9 μmol·L⁻¹ and isoform (PI3Kα, β, and δ) selectivity over 40-fold. Molecular docking, 100 ns molecular dynamics simulation and cellular thermal shift revealed that EuC binds to PI3Kγ. Further cell-based studies showed that EuC can inhibit the activation of spleen cells by inhibiting the PI3Kγ/NF-κb pathway and selectively induce the apoptosis of T cells through the PI3Kγ/AKT/mTOR/membrane apoptotic pathway. This study first demonstrated that EuC is a new selective PI3Kγ inhibitor with immunosuppressive effects, and the phloroglucinol-sesquiterpene adducts are the pharmacodynamic material basis of the fruit of *E. globulus* towards immunosuppression.

Appendix A. Supplementary data

The Supplementary data of this article can be acquired by E-mail to corresponding author.
Fig. 9 Effects of EuC and ON-01910 (an AKT/mTOR inhibitor) on the mitochondrial apoptotic pathway. (A) CCK-8 assay for cell viability. (B) The original bands of Bax, Bcl-2, and cleaved-caspase-3/9. (C–E) The quantitative analysis. *P < 0.01 vs the control group (n = 3).

References


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