Abietane Diterpenoids from *Plectranthus* spp. as a potential new class of Protein Kinase C Modulators

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Abietane Diterpenoids from *Plectranthus* spp. as a potential new class of Protein Kinase C Modulators

**Graphical Abstract**

**TARGET**

**Hit:**

**Human PKC-δ Regulatory domain**

*P. grandidentatus* Benth.

**Roy**

<table>
<thead>
<tr>
<th>Roy-Bz [μM]</th>
<th>2</th>
<th>2.5</th>
<th>3</th>
<th>3.5</th>
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<tbody>
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<td>0h</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>6h</td>
<td></td>
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<td>72h</td>
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<tr>
<td>96h</td>
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Abstract:
Cancer is one of the highest causes of death worldwide. Protein kinase C (PKC) is a family of kinases divided into three groups according to their regulatory domain structure and cofactors requirement for activation: classical, novel, and atypical PKCs. Recently, PKC family isoforms have been the focus of intense research, and recognized as therapeutic targets in anticancer drug development. Diterpenoids are commonly found in the Plectranthus spp., and have a widespread spectrum of biological activity, namely anticancer properties. The diterpenoid 7α-acetoxy-6β-hydroxyroyleanone (AHR) isolated from P. grandidentatus displays low cytotoxicity and the basic requirements approaches for the development of pharmaceutical formulations based on AHR as a lead. The results obtained revealed potent activators of PKC family proteins, namely: a selective activator of PKCd, the 7α-acetoxy-6β-benzoyloxy-12-O-benzoylroyleanone (Roy-Bz). The patented diterpenoid RoyBz was prepared using AHR as starting material. The results indicate that Roy-Bz targets drug resistant cancer stem cells, in HCT116 colon cancer cells, preventing tumor dissemination and recurrence. Moreover, these findings support a tumor suppressive function of PKCd in colon cancer. Overall, these results point to promising activators of PKCs with high potency and isoform-selectivity that may emerge from the exploitation of this new family of abietane diterpenoids.

Keywords: Cancer, PKC, Plectranthus, abietane
Role of PKCs in carcinogenesis is known since the late 1980s

- However:
  - poor understanding of isozymes-specific functions
  - limited availability of selective pharmacological modulators of PKC isozymes
  - compromised the clinical translation of PKC-targeting agents

**Figure**: Protein Kinase C (PKC) family regulatory and catalytic domains. DAG, diacylglycerol; PS, phosphatidylserine; PB1, Phox/Bem1; PSD, pseudosubstrate.

Protein Kinase C (PKC): Ca\(^{2+}\) dependent protein kinase activity

Milestone in the history of PKC

- **Identification of diacylglycerol** (DAG; Figure 1) as an endogenous activator of PKC

- **Discovery of the natural tumour-promoting phorbol esters** as PKC activators:
  - **PMA** (phorbol 12-myristate 13-acetate) also known as TPA (Figure 2)
    (extracted from the oil of the seed of the plant *Croton tiglium*)

- **Mimicked DAG** without generation of this unsaturated lipid

- **Phorbol esters competitively** act with DAG for the same binding site, and activate PKC in a similar manner

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PKC family

- Associated with a number of diseases, including cancer

- Most studied enzymes in biology (>58204 research papers)

- Knowledge in the PKC field:
  elucidate the molecular mechanisms involving PKC signalling in cancer progression

  promising therapeutic target in cancer

Inhibitors that directly interfere with kinases were described:
antifungal alkaloid staurosporine (elucidation of the role of PKC in several cellular functions)

Some natural products or analogues: clinical trials

Two approved compounds for specific cancer types (ingenol mebutate and bryostatin 1 in combination with paclitaxel)

PKCδ:

  associated with pro-apoptotic functions
  death mediator of chemotherapeutic agents and radiotherapy

PKCs isoforms: target of many natural products
few are selective to one isoform: not suitable to clinical use

Phorbol Esters

Bryostatins

Staurosporine Analogues

Ingenene Diterpenes

Daphnane Diterpenes

MISCELLANEOUS AND PROMISING AGENTS

Abietane Diterpenoids: Coleon U (A) and Carnosol (B)

Phenolic compounds

Resveratrol

Plectranthus genus as a valuable source of bioactive compounds

- **Plectranthus genus** (Lamiaceae family)
  - e.g. *Salvia officinalis* L. (Sage), *Melissa officinalis* L. (lemon balm)

- **Valuable source** of **bioactive natural products**, namely diterpenoids

- **Traditionally used:**
  - Tropical Africa, Asia and Austrália
  - Introduced in the New World, following the Portuguese Discoveries (XVI century): Africa and Brasil

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Development pharmaceutical formulations based on **AHR as a lead**: (Basic Requirements methods)

- Extraction optimization
- Characterization of its structural and thermal properties

**7α-acetoxy-6β-hydroxyroyleanone (AHR) from *P. grandidentatus***

Optical rotation = \([\alpha]^{21}_D = -2.70^\circ\)

Circular dichroism

Single crystal X-ray diffraction analysis
Optical microscopy image of the orthorhombic crystals of AHR

differential scanning calorimetry (DSC):
presence of two other polymorphs above room temperature

Extraction optimization, structural and thermal characterization of 7α-acetoxy-6β-hydroxyroyleanone

<table>
<thead>
<tr>
<th>Method</th>
<th>Solvent</th>
<th>Amount of AHR in P. grandidentatus (µg·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maceration extraction</td>
<td>Acetone</td>
<td>9.77</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>Acetone</td>
<td>8.04</td>
</tr>
<tr>
<td>Supercritical fluid extraction</td>
<td>CO₂</td>
<td>57.351</td>
</tr>
<tr>
<td>Decoction</td>
<td>H₂O</td>
<td>1.996</td>
</tr>
<tr>
<td>Infusion</td>
<td>H₂O</td>
<td>0.950</td>
</tr>
<tr>
<td>Microwave</td>
<td>H₂O</td>
<td>0.925</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>H₂O</td>
<td>0.928</td>
</tr>
</tbody>
</table>

**Polymorphism** may not perturb the development pharmaceutical formulations based on ARH

(at room temperature, forms I and II will quickly transform into form III)

3 enantiotropically related polymorphic forms (reversible): orthorhombic space group P21212 (crystal structure most stable phase up to 333.5 K)

Carlos E. S. Bernardes, Catarina Garcia, Filipe Pereira, Joana Mota, P. Pereira, Maria J. Cebola, Catarina P. Reis, M. Fátima M. Piedade, Manuel E. Minas da Piedade, Patrícia Rijo; Extraction optimization, structural and thermal characterization of the antimicrobial abietane 7α-acetoxy-6β-hydroxyroyleanone; Molecular pharmaceutics, 2018, in print.
Royleanone diterpenoids as potent activators of PKC family proteins

- Yeast-based screening assay*: search for modulators of PKC isoforms
- Small library of abietane derivatives: activate PKC isoforms from classical (α; β), novel (δ; ε) and atypical (ζ) subfamilies (Table 1).

Table 1. EC$_{50}$ values of compounds tested on individual PKC isoforms

<table>
<thead>
<tr>
<th>Compounds</th>
<th>PKα</th>
<th>PKCβI</th>
<th>PKCδ</th>
<th>PKCε</th>
<th>PKCζ</th>
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<tr>
<td>PMA</td>
<td>111.6±18.4</td>
<td>243.2±69.1</td>
<td>573.8±36.7</td>
<td>1678±46.48</td>
<td>-</td>
</tr>
<tr>
<td>ARA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>205.4±32.6</td>
</tr>
<tr>
<td>Roy</td>
<td>350±42</td>
<td>423±67</td>
<td>ND</td>
<td>994±63</td>
<td>4113±159</td>
</tr>
<tr>
<td>Ac-Roy-Pr2</td>
<td>195±16</td>
<td>229±21</td>
<td>325±49</td>
<td>770±46</td>
<td>ND</td>
</tr>
<tr>
<td>Roy-Bz</td>
<td>ND</td>
<td>ND</td>
<td>107.53</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DeRoy</td>
<td>15±1.9</td>
<td>0.97±4.34</td>
<td>3.1±60</td>
<td>5.8±0.70</td>
<td>43.8±2.32</td>
</tr>
</tbody>
</table>

EC$_{50}$ values were considered the concentration of compound that caused 50% of the maximal growth inhibition caused by the positive controls (PMA, for cPKCs and nPKCs; arachidonic acid, ARA, for PKCζ), which was set as 100%. Data are mean ± SEM of four independent experiments. ND: non determinable (when the maximal response achieved was lower than 50% growth inhibition).

RoyBz: PKCδ-selective activator in colon cancer therapy

**Research for more selective PKC modulators**

Roy-Bz* (7α-acetoxy-6β-benzoyloxy-12-O-benzoylroyleanone):

*semi-synthesis* from 7α-acetoxy-6β-hydroxyroyleanone (AHR)

The first small molecule PKCδ-selective activator

potently inhibited the proliferation of colon cancer cells

a novel *anticancer drug candidate*, particularly in *colon cancer therapy*

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Roy-Bz: selective activator of PKCδ that binds to the C1 domain

Potential binding mode of Roy-Bz to PKCδ (molecular docking studies)

In vitro kinase assay with recombinant PKCs; increase of PKC activity by $10^{-4}$ μM PMA/ARA and $10^{-4}$ μM Roy-Bz

Predicted binding models support the Roy-Bz binding to the PKCδ C1 domain (like 13-acetylpohorbal (PRB; positive control))

Roy-Bz inhibits the proliferation of colon cancer cells

**Selectivity** of Roy-Bz as PKCδ activator: impact of Roy-Bz on the translocation of PKCα, PKCδ and PKCε in human HCT116 cells.

**Dose-dependent reduction of cell growth** (IC50 values of 0.58 ± 0.05 μM for HCT116, 1.50 ± 0.06 μM for HT-29, and 1.08 ± 0.03 μM for SW-837; n=5).

Roy-Bz inhibits the proliferation of colon cancer cells

In HT-29 and SW-837 cells the Roy-Bz growth inhibitory effect was associated with G2/M-phase cell cycle arrest and apoptosis.

In HCT116 cells the Roy-Bz-induced growth inhibition was only mediated by apoptosis.

In HCT116 cells, the induction of apoptosis by Roy-Bz was further reinforced by the occurrence of caspase-3 and PARP cleavage, increase of the pro-apoptotic p53 and Bax levels, and a reduction in the levels of the anti-apoptotic proteins Bcl-2 and survivin.

**Roy-Bz inhibits the proliferation of colon cancer cells**

The involvement of the mitochondrial pathway in Roy-Bz-induced apoptosis was also evidenced by the increase of mitochondrial ROS generation and Δψ<sub>m</sub> dissipation.

Explore the antitumor activity of Roy-Bz: system that more closely resembles the *in vivo* features of the tumor microenvironment

highly enriched in a small population of cancer stem cells (CSCs)

Spheroid-formation -colonosphere culture model - was generated from HCT116 cells (valuable tool for assessment and expansion of stem cells in colon cancer)

notable dose-dependent reduction in colonosphere formation ability by Roy-Bz, with an abolishment of colonosphere formation at 1 \( \mu \text{M} \) Roy-Bz

PKCδ-selective activator in colon cancer therapy

Roy-Bz pro-apoptotic and anti-migratory activity in HCT116 cancer cells is mediated by PKCδ-selective activation

Roy-Bz is non-genotoxic in human cancer and normal cells and has in vivo PKCδ-dependent antitumor activity (in human xenograft mouse models) with no apparent toxic side effects

Molecular Docking with human PKC-δ

TARGET
Selection of the most appropriate protein structure (PDB)

Human PKC-δ Regulatory domain

1PTR

AIM
Assess which substituent groups could enhance the PKC activity of each position C-12 and C-6

3rd International Electronic Conference on Medicinal Chemistry
1-30 November 2017
Docking Studies
~ 250 Compounds screened

PKC modulation:
position 6 can bear high diversity of substituents
position 12 requires small groups
Furthers docking studies for PKCs selectivity

Hit:

3 Hydrogen bonds:
Acetoxyl group and =O (C14) to Glutamine 257
Propionic group to Glycine 253

Roy
Conclusions

Search for new drugs: Natural products

*Plectranthus* genus (*Lamiaceae* family): source of bioactive lead compounds

Roy-Bz: the first small molecule PKCδ-selective activator, with encouraging clinical application in colon cancer therapy

opens the way to a new era on PKC biology and pharmacology

elucidation of the structural requirements underlying its selectivity to PKCδ:

will be crucial to the structure-based design of isozymes-selective agents

Promising modulators of PKCs with high potency and isoform-selectivity:

may emerge from the exploitation of this family of compounds

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