3rd International Electronic Conference on Medicinal Chemistry
1-30 November 2017
chaired by Dr. Jean Jacques Vanden Eynde
sponsored by pharmaceuticals

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

Patrícia Rijo[1,2*], Vera M. S. Isca[1,2], Epole Ntungwe[1], Cláudia Bessa[3], Carlos A.M. Afonso[2], Lucília Saraiva[3]

[1] Center for Research in Biosciences & Health Technologies (CBIOS), Universidade Lusófona de Humanidades e Tecnologias, 1749-024 Lisboa, Portugal,
[2]Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal,
[3]LAQV/REQUIMTE, Laboratório de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal
* Corresponding author: patricia.rijo@ulusofona.pt

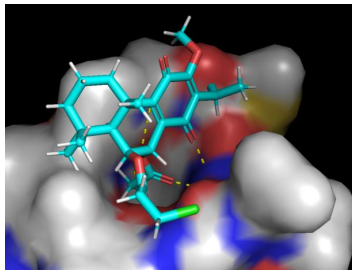
cbios UNIVERSIDADE LUSÓFONA
UNIVERSIDADE LUSÓFONA
iMed. ULisboa Research Institute for Medicines
LAQV @ REQUIMTE

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

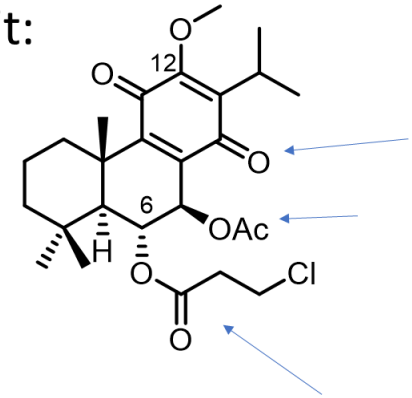
Graphical Abstract

Human PKC-δ
Regulatory domain

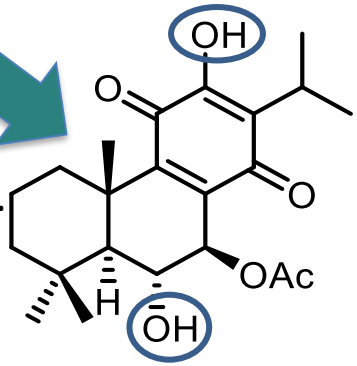
TARGET



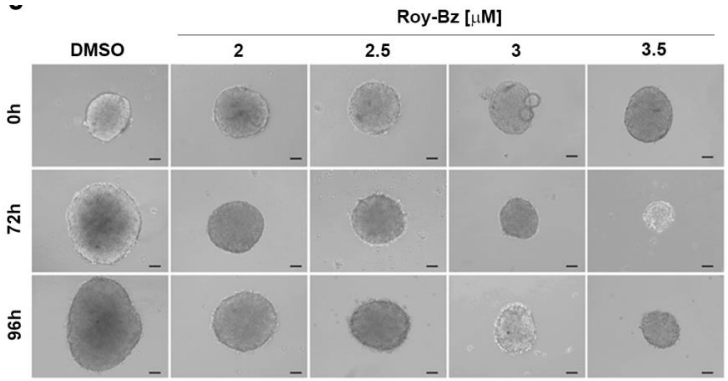
Hit:



P. grandidentatus Benth.



Roy



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**

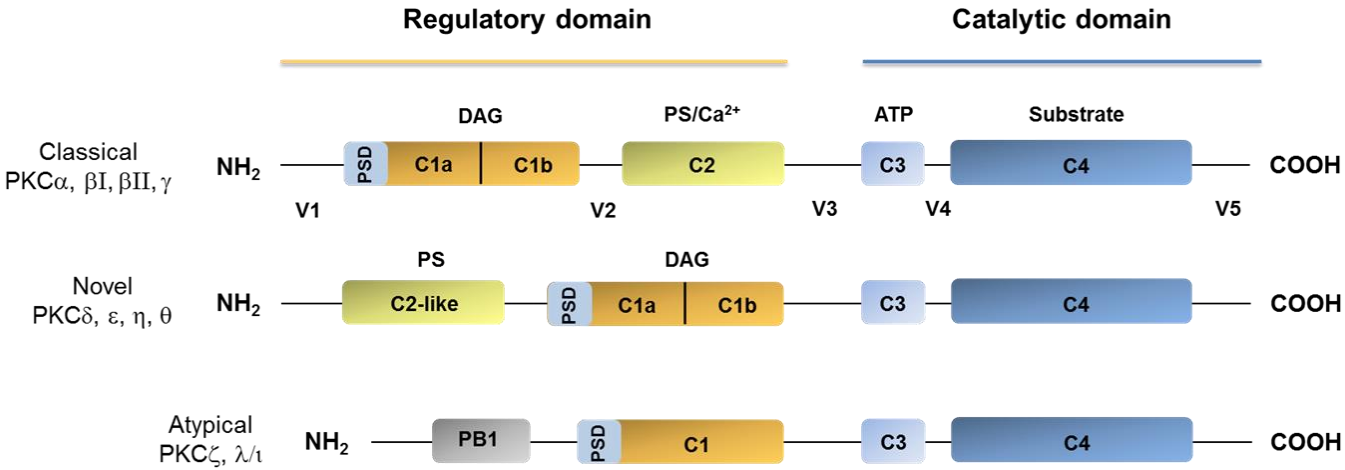


Figure: Protein Kinase C (PKC) family regulatory and catalytic domains. DAG, diacylglycerol; PS, phosphatidylserine; PB1, Phox/Bem1; PSD, pseudosubstrate.

D. Matias, C. Bessa, M.F. Simões, C.P. Reis, L. Saraiva, P. Rijo*, Natural Products as Lead Protein Kinase C Modulators for Cancer Therapy, in: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, 2016, pp. 45–79

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**

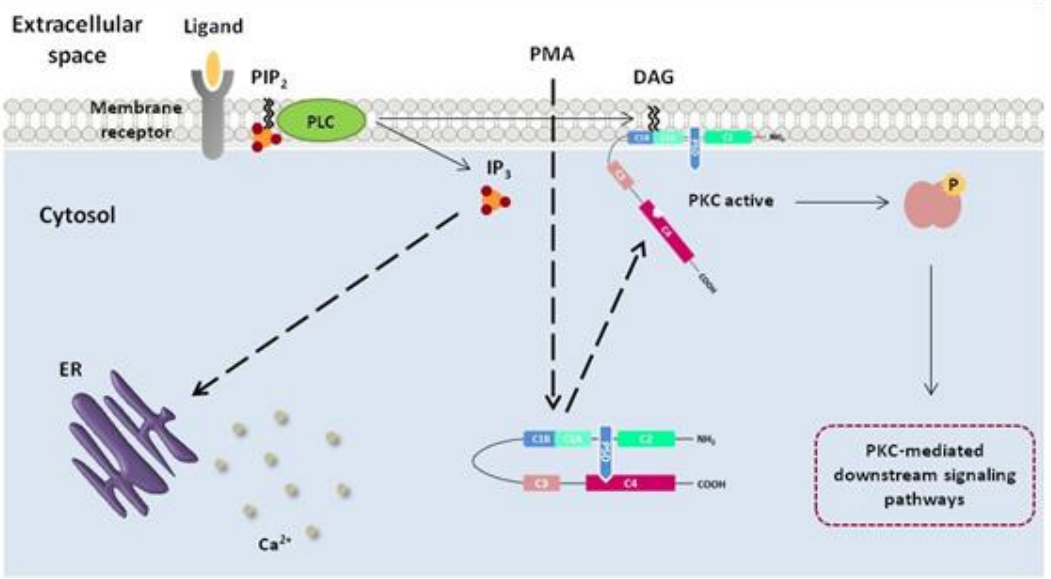


Figure 1: General mechanism of activation of PKCs by DAG and PMA.

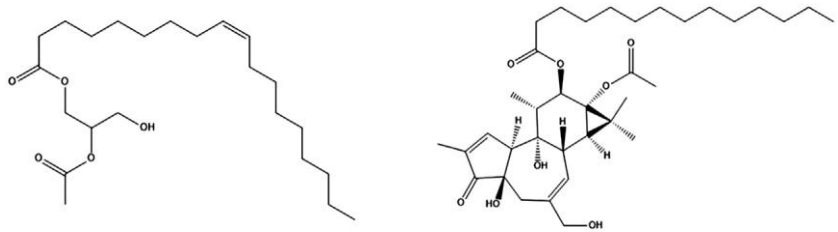


Figure 2: Diacylglycerol (**DAG**) and phorbol 12-myristate 13-acetate (**PMA**) structures

D. Matias, C. Bessa, M.F. Simões, C.P. Reis, L. Saraiva, P. Rijo*, Natural Products as Lead Protein Kinase C Modulators for Cancer Therapy, in: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, 2016, pp. 45–79

PKC family

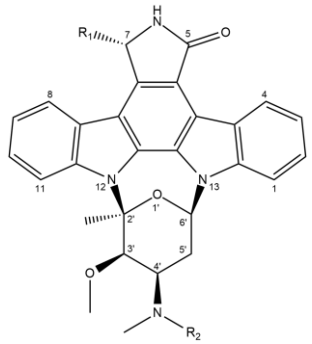
- Associated with a **number of diseases, including cancer**

- 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



	R_1	R_2
Staurosporine	-H	-H

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)

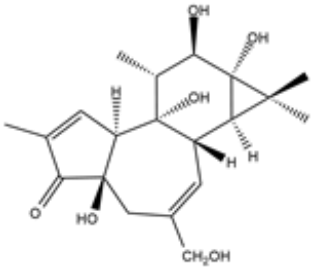
PKCδ:

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

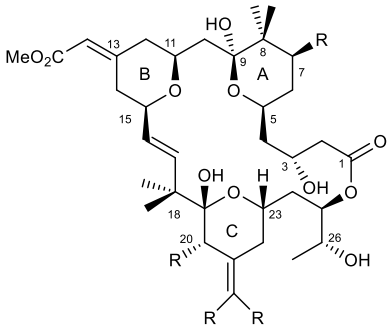
D. Matias, C. Bessa, M.F. Simões, C.P. Reis, L. Saraiva, P. Rijo*, Natural Products as Lead Protein Kinase C Modulators for Cancer Therapy, in: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, 2016, pp. 45–79

PKCs isoforms: target of many natural products

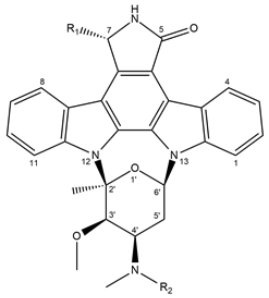
few are selective to one isoform: not suitable to clinical use



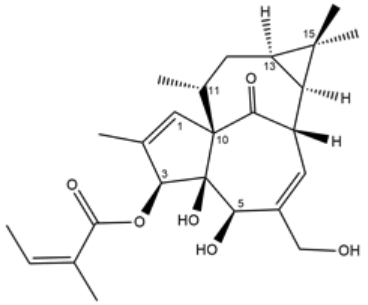
Phorbol Esters



Bryostatins

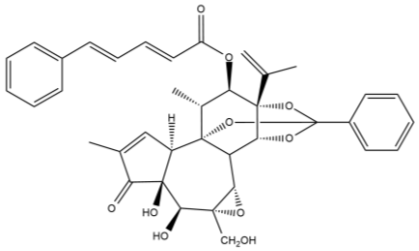


Staurosporine Analogues

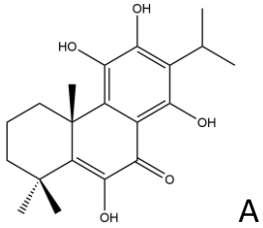


Ingenene Diterpenes

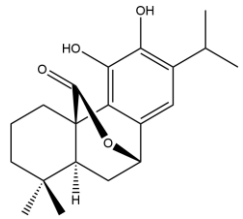
MISCELLANEOUS AND PROMISING AGENTS



Daphnane Diterpenes



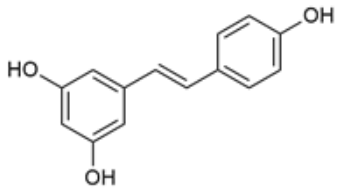
A



B

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

Phenolic compounds



Resveratrol

D. Matias, C. Bessa, M.F. Simões, C.P. Reis, L. Saraiva, P. Rijo*, Natural Products as Lead Protein Kinase C Modulators for Cancer Therapy, in: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, 2016, pp. 45–79

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

Plants obtained from South Africa and cultured in Portugal (*Instituto Superior de Agronomia de Lisboa*)

C. Garcia, C. Teodósio, C. Oliveira, C. Oliveira, A. Díaz-Lanza, C. P. Reis, N. Duarte, P. Rijo. Naturally occurring *Plectranthus*-derived abietane diterpenes with antitumoral activities. *Current Pharmaceutical Design*. 2019, 24(36): 4207 – 4236.

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

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

  - **Extraction optimization**

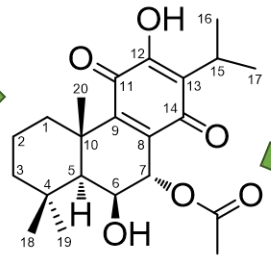
  - **Characterization of its structural and thermal properties**

Optical rotation = $[\alpha]_D^{21}$ −2.70º

Circular dichroism

Plectranthus grandidentatus

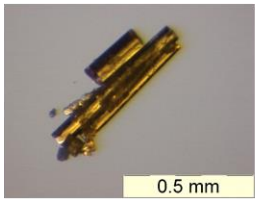
ISOLATION



7α-acetoxy-6β-hydroxyroyleanone

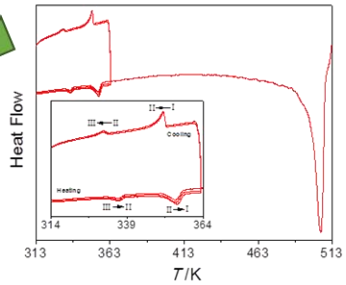
(AHR)

SCXRD



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

DSC



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

C. E. S. Bernardes, C. Garcia, F. Pereira, J. Mota, P. Pereira, M. J. Cebola, C. P. Reis, M. F. M. Piedade, M. E. Minas da Piedade, P. Rijo; Extraction optimization, structural and thermal characterization of the antimicrobial abietane 7a-acetoxy-6ß-hydroxyroyleanone; *Molecular Pharmaceutics* (2018) 2;15(4):1412-1419.

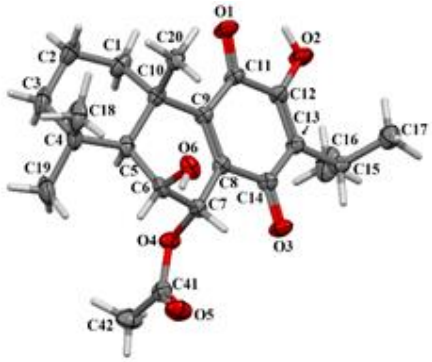
Extraction optimization, structural and thermal characterization of 7α-acetoxy-6β-hydroxyroyleanone

Extraction optimization

Method	Solvent	Amount of AHR in *P. grandidentatus* (μg·mg^{-1})
Maceration extraction	Acetone	9.77
Ultrasound	Acetone	8.04
Supercritical fluid extraction	CO_2	57.351
Decoction	H_2O	1.996
Infusion	H_2O	0.950
Microwave	H_2O	0.925
Ultrasound	H_2O	0.928

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

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



Molecular structure of 7α-acetoxy-6β-hydroxyroyleanone (AHR) with the atom labelling scheme

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 7a-acetoxy-6ß-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).

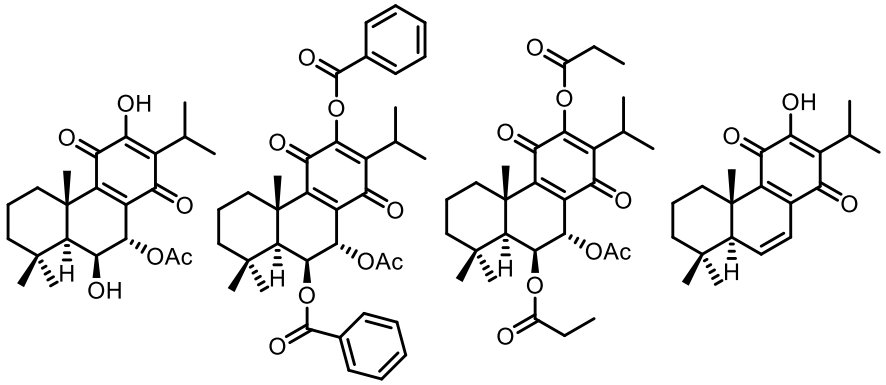


Table 1. EC_{50} values of compounds tested on individual PKC isoforms

Compounds	EC_{50} (nM)				
	PKα	**PKCβI**	**PKCδ**	**PKCε**	**PKCζ**
PMA	111,6±18,4	243.2±69,1	573,8±36,7	1678±46,48	-
ARA	-	-	-	-	205,4±32.6
Roy	350±42	423±67	*ND*	994±63	4113±159
Ac-Roy-Pr2	195±16	229±21	325±49	770±46	*ND*
Roy-Bz	*ND*	*ND*	107.53	*ND*	*ND*
DeRoy	**15±1.9**	**0.97±4.34**	**3.1±60**	**5.8±0.70**	**43.8±2.32**

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).

*Coutinho *et al.,* Biochem Pharmacol. 2009, 78:449-459

RoyBz: PKCδ-selective activator in colon cancer therapy

Research for more selective PKC modulators

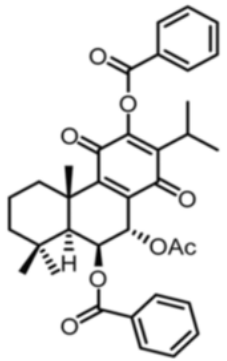
Roy-Bz* (7α-acetoxy-6β-benzoyloxy-12-*O*-benzoylroyleanone):

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**

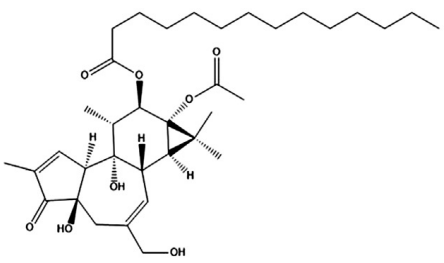


Roy-Bz*

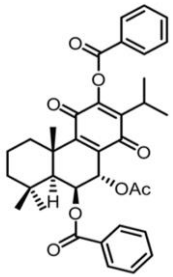
Rijo P, Simões MF, Francisco AP, Rojas R, Gilman RH, Vaisberg AJ, et al. (2010). *Chem Biodivers* 7: 922-932.

*Cláudia Bessa, Joana Soares, Liliana Raimundo, M. Fátima Simões, Jorge Gonçalves, Patrícia Rijo, Lucília Saraiva**. International Patent** nº 109140: "Roy-Bz: A small molecule selective activator of Protein Kinase Cδ", PCT/IB2017/050633, 2016.

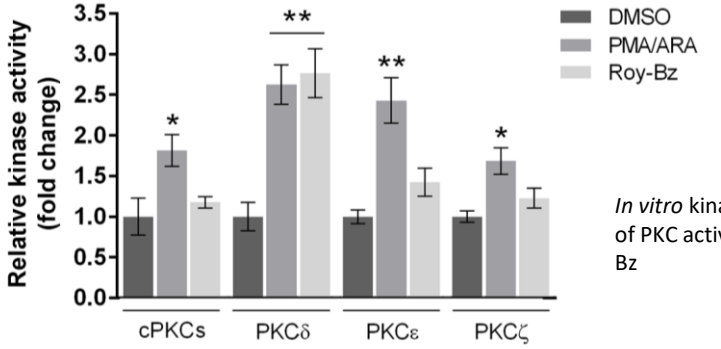
Roy-Bz: selective activator of PKCδ that binds to the C1 domain



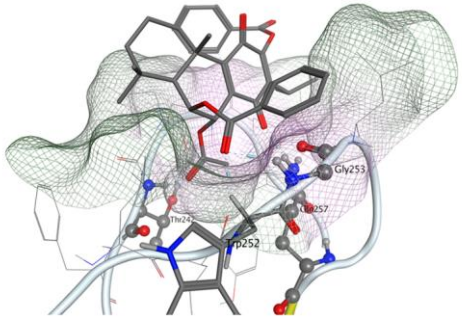
PMA



Roy-Bz

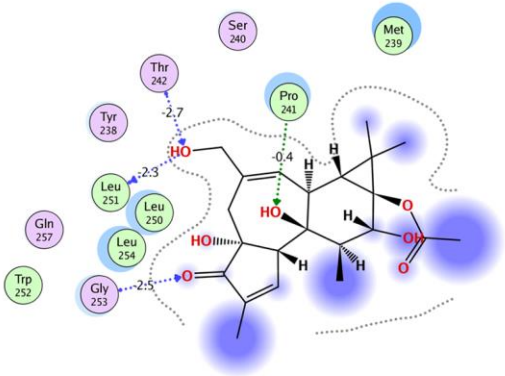


In vitro kinase assay with recombinant PKCs; increase of PKC activity by 10^{-4} µM PMA/ ARA and 10^{-4} µM Roy-Bz

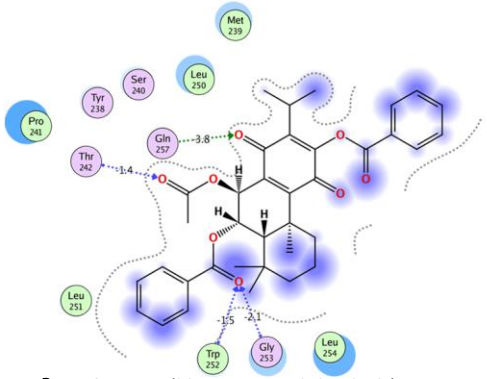


Potential **binding mode** of Roy-Bz to PKCδ (**molecular docking studies**)

PRB



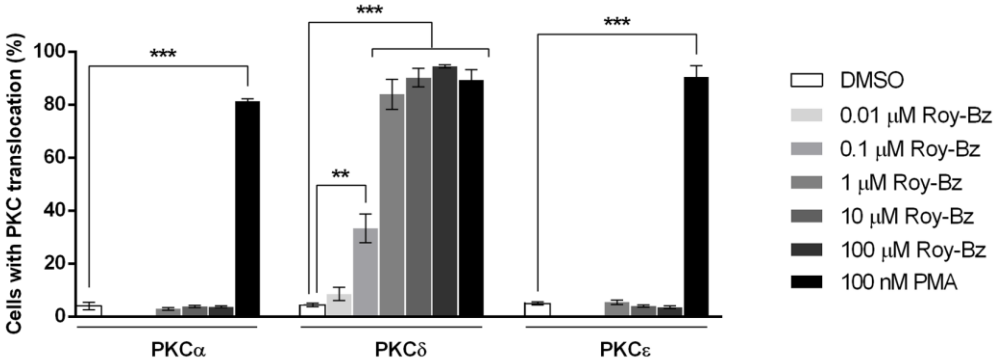
Roy-Bz



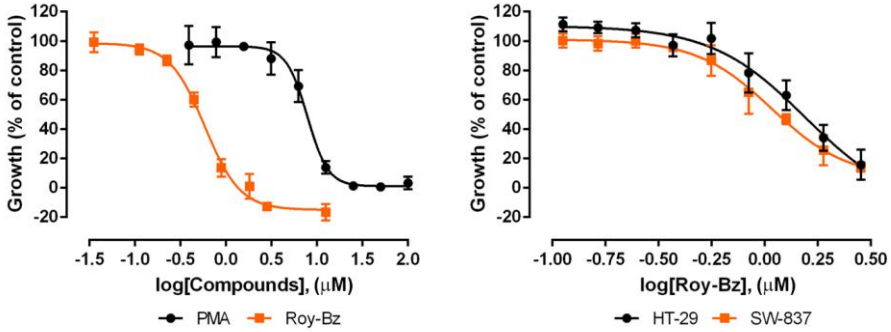
Predicted binding models support the **Roy-Bz binding to the PKCδ C1 domain** (like 13-acetylphorbol (PRB; **positive control**)

Bessa C, Soares J, Raimundo L, Loureiro J B, Gomes C, Reis F, Soares M, Santos D, Dureja C, Chaudhuri SR, Lopez-Haber C, Kazanietz MG, Gonçalves J, Simões MF, Rijo P, Saraiva L (2018). Cell death & disease. 18;9(2):23. doi: 10.1038/s41419-017-0154-9

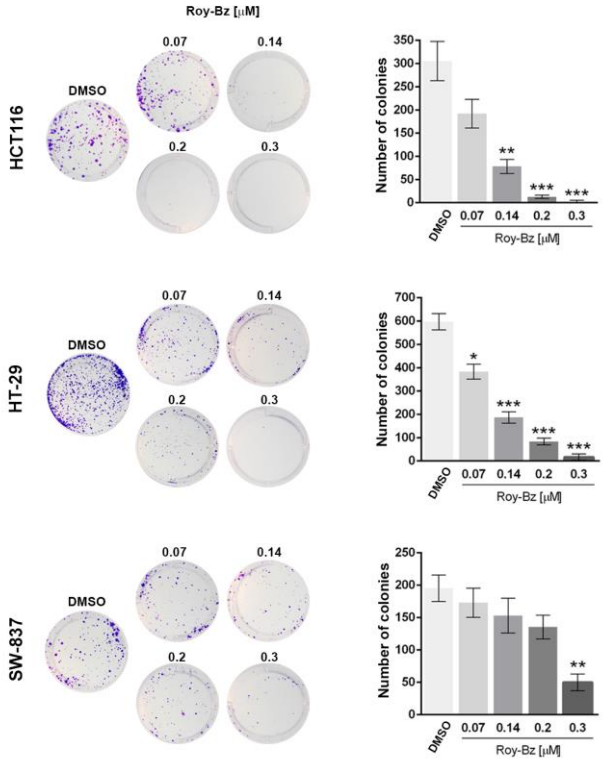
Roy-Bz inhibits the proliferation of colon cancer cells



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



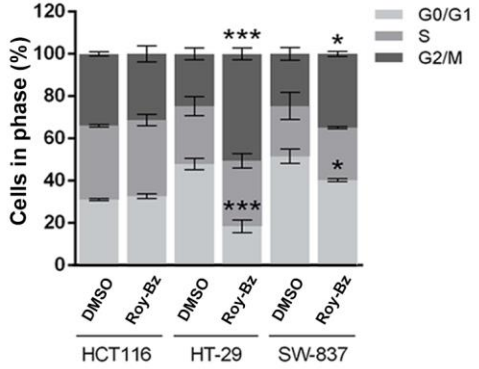
Dose-dependent reduction of cell growth (IC_{50} values of 0.58 ± 0.05 μM for HCT116, 1.50 ± 0.06 μM for HT-29, and 1.08 ± 0.03 μM for SW-837; *n*=5).



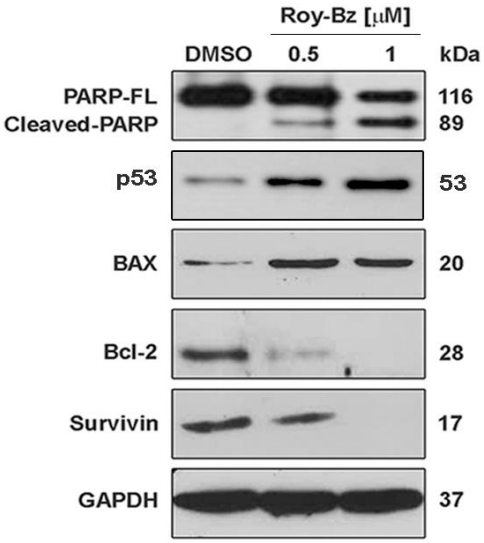
Inhibitory effect of Roy-Bz on cell proliferation/viability of colorectal cancer cells by assessing the **colony forming ability**

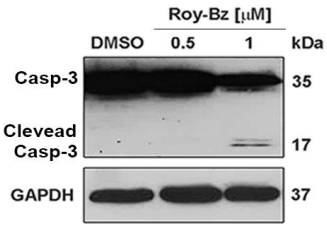
Bessa C, Soares J, Raimundo L, Loureiro J B, Gomes C, Reis F, Soares M, Santos D, Dureja C, Chaudhuri SR, Lopez-Haber C, Kazanietz MG, Gonçalves J, Simões MF, Rijo P, Saraiva L (2018). Cell death & disease. 18;9(2):23. doi: 10.1038/s41419-017-0154-9

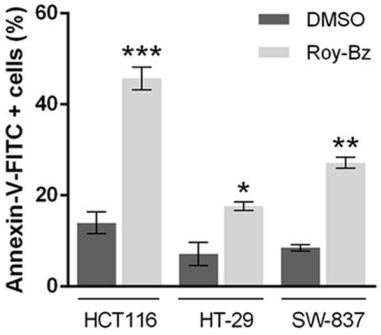
Roy-Bz inhibits the proliferation of colon cancer cells



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**



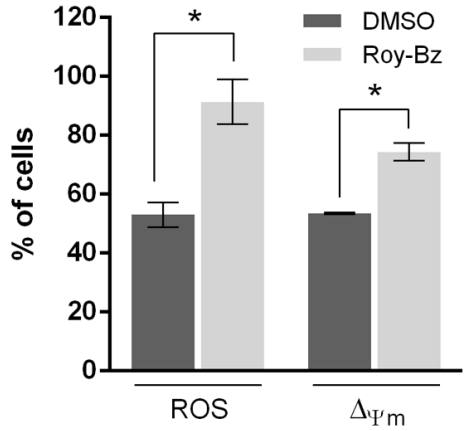




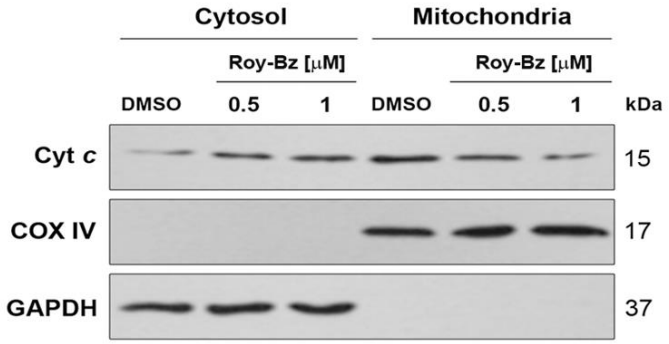
In HCT116 cells, the **induction of apoptosis** by Roy-Bz was further reinforced by the **occurrence of caspase-3** and **PARP cleavage**, **increase of the pro-apoptotic p53** and **Bax levels**, and a reduction in the levels of the anti-apoptotic proteins Bcl-2 and survivin

Bessa C, Soares J, Raimundo L, Loureiro J B, Gomes C, Reis F, Soares M, Santos D, Dureja C, Chaudhuri SR, Lopez-Haber C, Kazanietz MG, Gonçalves J, Simões MF, Rijo P, Saraiva L (2018). Cell death & disease. 18;9(2):23. doi: 10.1038/s41419-017-0154-9

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 $\Delta\psi_m$ dissipation



release of cytochrome c to cytosol

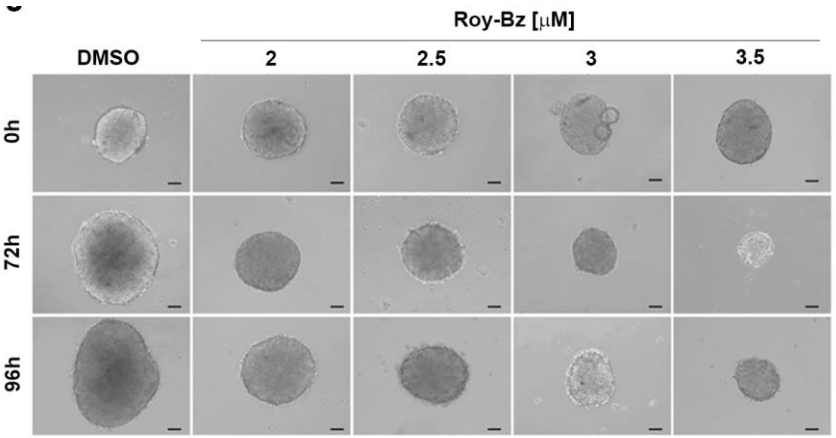
Bessa C, Soares J, Raimundo L, Loureiro J B, Gomes C, Reis F, Soares M, Santos D, Dureja C, Chaudhuri SR, Lopez-Haber C, Kazanietz MG, Gonçalves J, Simões MF, Rijo P, Saraiva L (2018). Cell death & disease. 18;9(2):23. doi: 10.1038/s41419-017-0154-9

Roy-Bz inhibits the proliferation of colon cancer cells

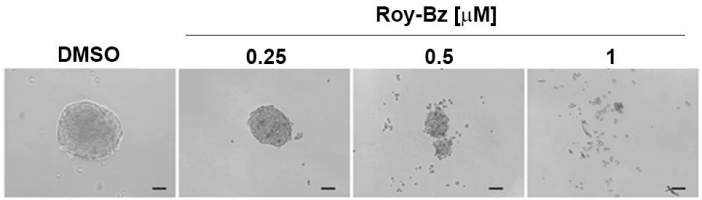
Explore the antitumor activity of Roy-Bz:
system that more **closely resembles** the ***in vivo*** **features** of the **tumor microenvironment**

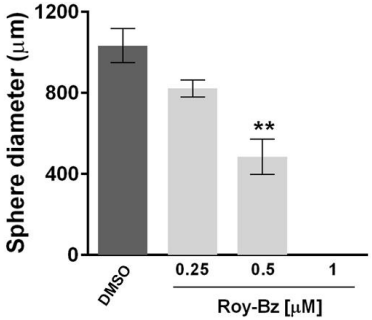
highly enriched in a small population of **cancer stem cells** (CSCs)

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 μM Roy-Bz

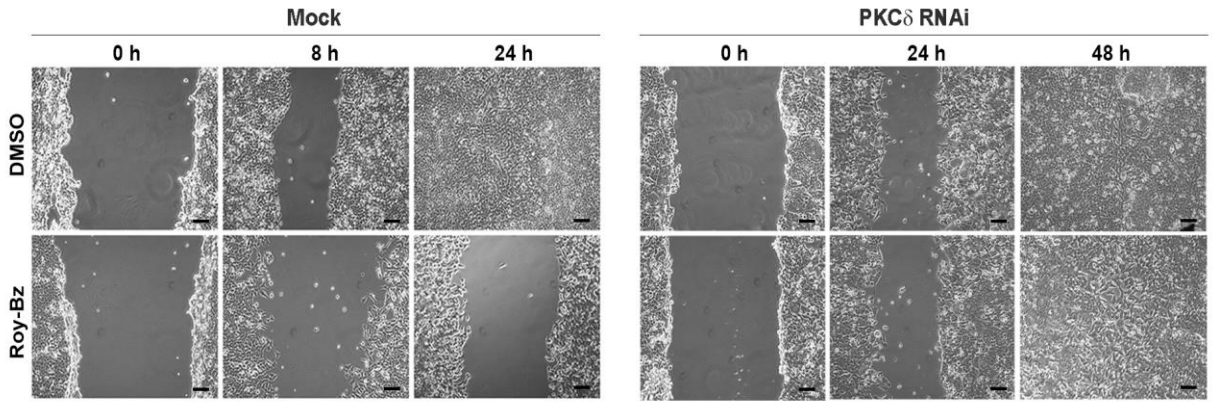




Bessa C, Soares J, Raimundo L, Loureiro J B, Gomes C, Reis F, Soares M, Santos D, Dureja C, Chaudhuri SR, Lopez-Haber C, Kazanietz MG, Gonçalves J, Simões MF, Rijo P, Saraiva L (2018). Cell death & disease. 18;9(2):23. doi: 10.1038/s41419-017-0154-9

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**

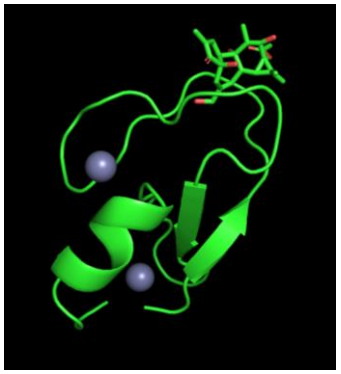
Bessa C, Soares J, Raimundo L, Loureiro J B, Gomes C, Reis F, Soares M, Santos D, Dureja C, Chaudhuri SR, Lopez-Haber C, Kazanietz MG, Gonçalves J, Simões MF, Rijo P, Saraiva L (2018). Cell death & disease. 18;9(2):23. doi: 10.1038/s41419-017-0154-9

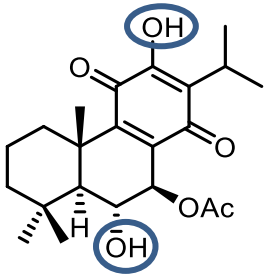
Molecular Docking with human PKC-δ

TARGET
Selection of the most appropriate protein structure (PDB)

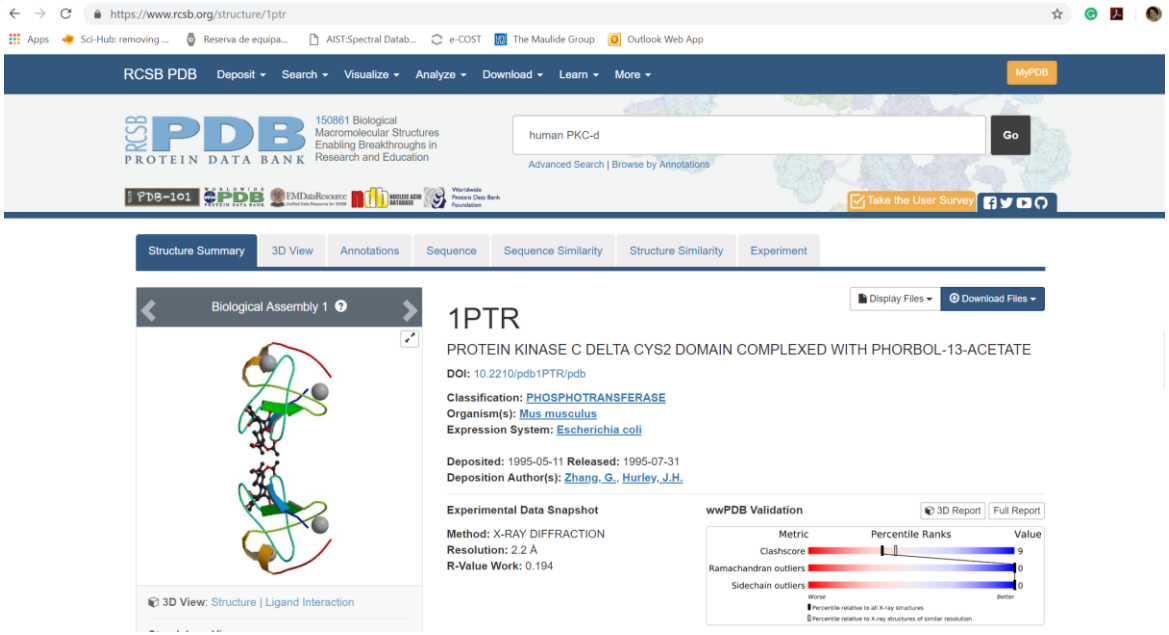
Human PKC-δ Regulatory domain

1PTR





Roy

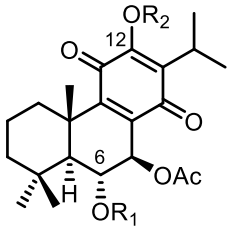


AIM
Assess which substituent groups could enhance the PKC activity of each position C-12 and C-6

Docking Studies

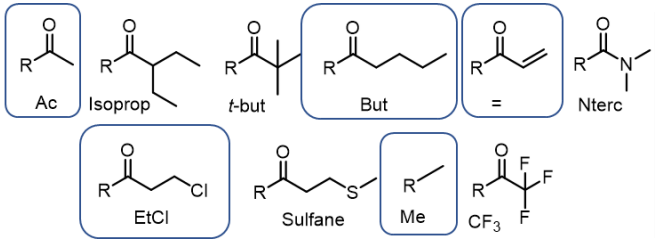
~ 250

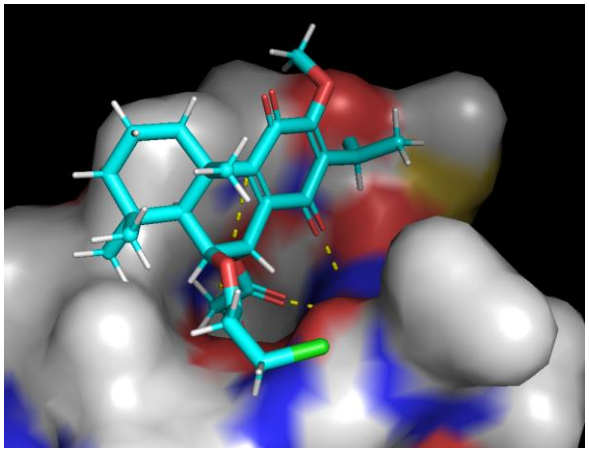
Compounds screened



Roy

R =



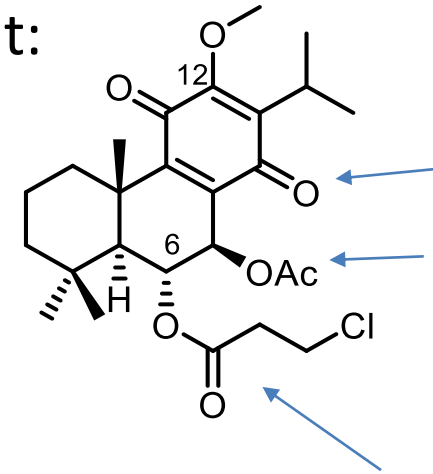


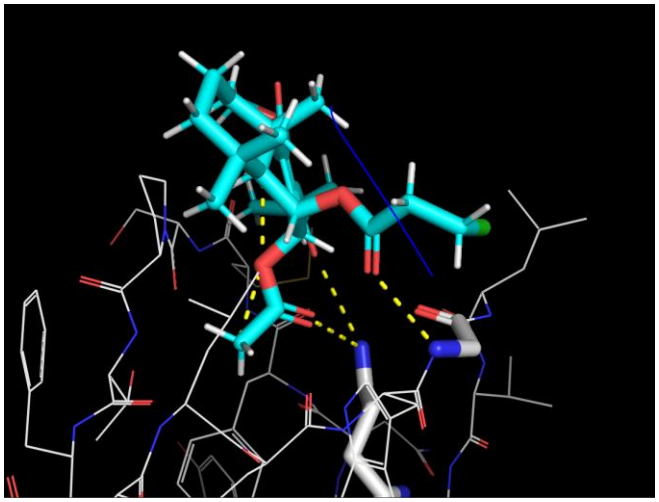
3 Hydrogen bonds:

Acetoxyl group and =O (C14) to Glutamine 257

Propionic group to Glycine 253

Hit:





PKC modulation:

position 6 can bear **high diversity of substituents**

position 12 requires **small groups**

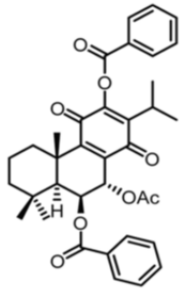
Furthers docking studies for PKCs **selectivity**

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**



Roy-Bz*

*Cláudia Bessa, Joana Soares, Liliana Raimundo, M. Fátima Simões, Jorge Gonçalves, Patrícia Rijo, Lucília Saraiva**. International Patent** nº 109140: "Roy-Bz: A small molecule selective activator of Protein Kinase Cδ", PCT/IB2017/050633, 2016.

Acknowledgments

Vera Isca
Epole Ntungwe
(Lisbon, Portugal)

Cláudia Bessa
Joana Soares
Liliana Raimundo
Joana B. Loureiro
Daniel Santos
Lucília Saraiva
(Porto, Portugal)

Célia Gomes
Flávio Reis
(Coimbra, Portugal)

Saumya Ray Chaudhuri
(India)

Marcelo G. Kazanietz
(USA)

Carlos Afonso
Daniel Santos

Jorge Gonçalves
(Porto, Portugal)

Funding

UID/DTP/04567/2019 –
CBIOS/PRUID/BI1/2017