

# PEP005, A NOVEL INGENOL ANGELATE MEDIATES APOPTOSIS IN HUMAN CANCER CELL LINES BY ACTIVATION OF P38 AND MAPK PATHWAYS, VIA A PKC-DEPENDENT MECHANISM

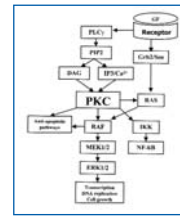
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## INTRODUCTION

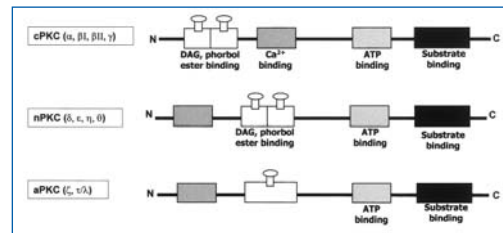
### Protein kinase C family

Protein kinases C (PKC) belongs to a family of serine-threonine kinases and are cytosolic nucleotide-independent enzymes that phosphorylate serine and threonine residues in many target proteins. Protein kinase C (PKC) family enzymes participate in several cell signaling pathways by controlling proliferation, differentiation, senescence, invasion, and apoptosis both in normal and cancer cells.



The protein kinase C (PKC) enzyme family has been recognized to comprise more than 12 isoforms that have been divided into three groups based on their interactions with calcium and diacylglycerol (DAG).

Classic PKCs, including isoforms  $\alpha$ ,  $\beta$ ,  $\beta$ II and  $\gamma$ , require both calcium and DAG for activation. Novel PKCs, including  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ , are independent of calcium but require DAG for activation. Atypical PKCs, including  $\lambda$  and  $\zeta$ , are independent of both calcium and DAG. Each isoform plays a different role in cell growth, proliferation, differentiation or apoptosis.



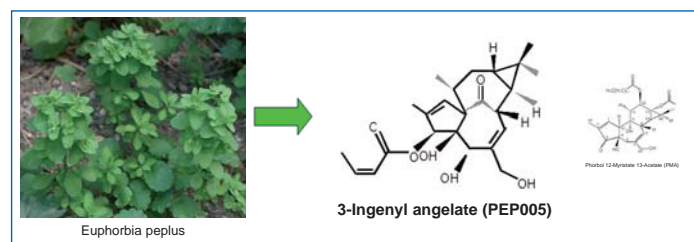
### PEP005 is a PKC modulator

PEP005 is an ingenol angelate extracted and purified from Euphorbia peplus. Currently, PEP005 is being developed as (1) a topical treatment for actinic keratoses and (2) basal cell skin carcinoma and as a systemic treatment for leukemia (3).

At relatively high concentrations of PEP005 mitochondrial disruption and cell death were associated with PEP005 cytotoxicity.

PEP005 has been shown to be a potent activator of Protein Kinase C a family of signaling enzymes that regulate several key cellular processes including proliferation, differentiation and apoptosis.

### Chemical structure of PEP005 and PMA



## AIMS OF THE STUDY

- Characterizations of a panel of human tumor cell lines
  - Representative relevant tumor types for further drug development
  - To evaluate the expression of PKC isoforms and their phosphorylation levels in the panel of selected cancer cell lines
- To establish concentrations of PEP005 associated with :
  - Cell cycle arrest
  - Apoptosis and necrosis
- To evaluate PEP005 effects on intracellular signaling in sensitive and resistant cancer cell lines to better characterize its molecular mechanism of action

## MATERIAL AND METHODS

Molecular characteristics of tumor cancer cell lines used in our study

Cell line	p53	MDR	MMR	HER2/neu	EGFR	PKC $\delta$
<b>Colon</b>						
HT29	mut	Low	High	Low	High	High
HCT116	wt	Low	Low	Low	High	High
COLO205	mut	Low	High	High	High	High
HCC2998	mut	Low	Low	High	High	High
<b>Breast</b>						
MCF7	wt	Low	Low	Low	Low	Low
MDA-MB-435	mut	Low	Low	High	Low	High
<b>Ovarian</b>						
OVCAR3	mut	Low	High	High	High	Low
IGROV1	wt	Low	Low	High	High	Low
<b>Lung</b>						
Hop62	mut	High	High	Low	Low	Low
Hop92	mut	Low	Low	High	High	High

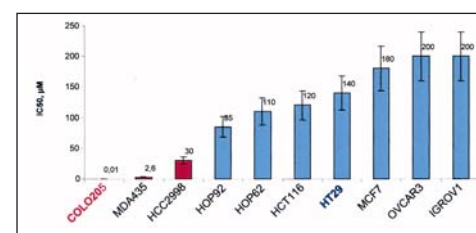
**MTT Assay** : Cells seeded at 2\*10<sup>3</sup>/well in 96-well plates and treated with different concentration of drugs for various times, incubated with 0.4 mg/ml MTT for 4 hours at 37°C. After incubation, the supernatant was discarded, the cell pellet was resuspended in 0.1 ml of DMSO and the absorbance was measured at 560 nm. Growth inhibition curves were plotted as a percentage of untreated control cells.

**Cell cycle, apoptosis and necrosis analysis** : Cells treated with various concentrations of PEP005 at various time-points were recovered, washed with PBS, fixed in 70% ethanol and stored at +4°C until use. Dehydrated in PBS, incubated for 30 min at 37°C with 1mg/ml RNase A with 12.8g/ml propidium iodide in the citrate buffer at +4°C. The cell cycle distribution and percentage of apoptotic cells were determined with FACSscan flow cytometer. Apoptosis and necrosis were tested with annexin V/PI kit and FACS analysis.

**Immunostaining** : Expressions of PKCs and MAPK was tested in sensitive and resistant cells. Cells treated with various concentrations of PEP005 were washed and lysed, equal amounts of proteins were electrophoresed on 10% SDS-PAGE and transferred PVDF membrane. Membranes were blocked, incubated with specific antibodies and revealed by peroxidase secondary antibody. The immunostaining was visualized using ECL.

## RESULTS

### 1 - PEP005 Cytotoxicity

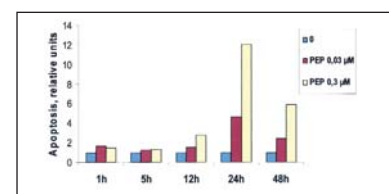


PEP005 displayed cytotoxic effects against human colon COLO205 and HCC2998 cells, and MDA-MB-435 breast cancer cells, and COLO205 being the most sensitive.

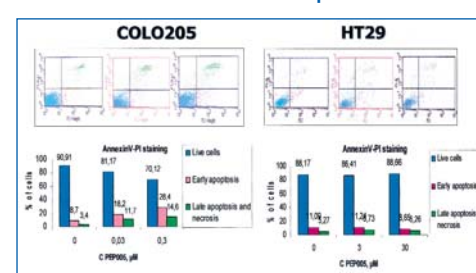
The human colon HT-29 cancer cell line was selected as being primarily resistant to PEP005 compared to COLO-205 that displays sensitivity.

### 2 - Apoptosis and necrosis induction

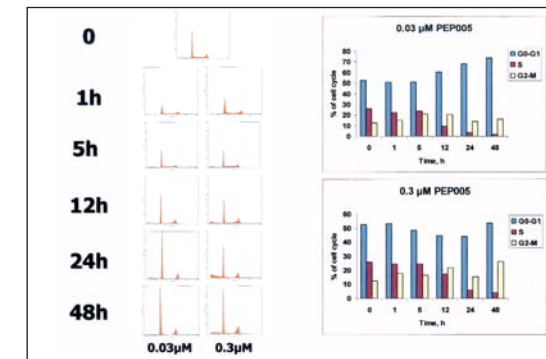
Sub-G1 DNA content, PI-staining



Apoptosis and necrosis induction after 24h PEP005 exposure

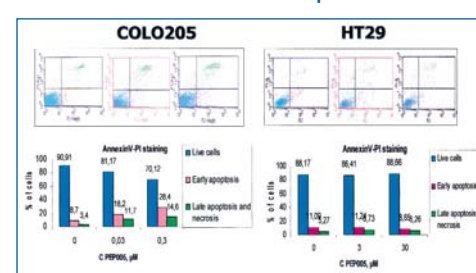


Cell cycle changes

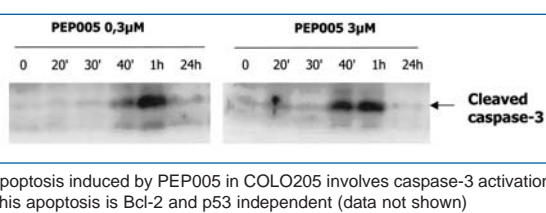


PEP005 exposure led to a dose dependent accumulation of cells in G0-G1 phase with an almost a complete block of G1/S transition.

Apoptosis and necrosis induction after 24h PEP005 exposure



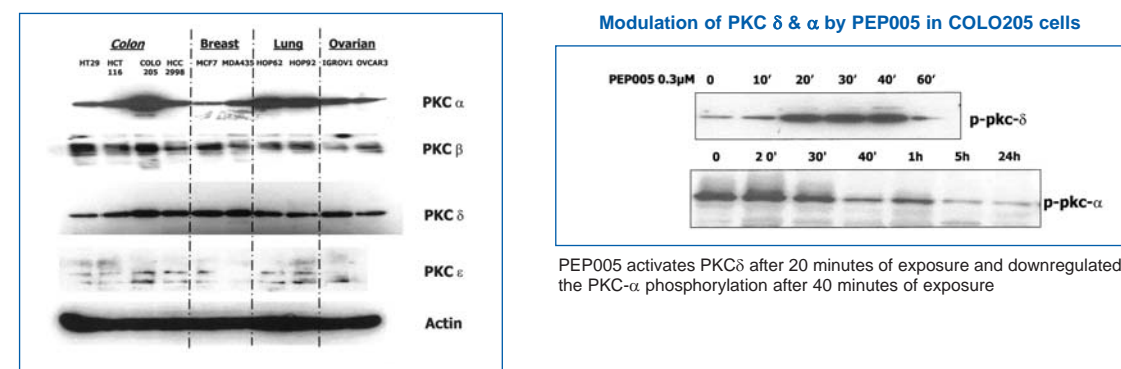
Caspase-3 activation by PEP005 in COLO205



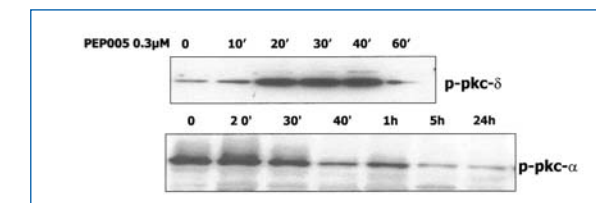
Apoptosis induced by PEP005 in COLO205 involves caspase-3 activation. This apoptosis is Bcl-2 and p53 independent (data not shown)

## RESULTS

### 3 - Characterization of PKC isoenzymes in a panel of humancancer cell lines

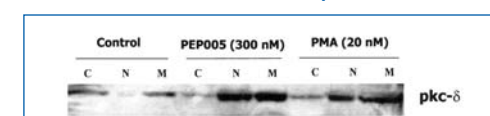


Modulation of PKC  $\delta$  &  $\alpha$  by PEP005 in COLO205 cells



PEP005 activates PKC $\delta$  after 20 minutes of exposure and downregulated the PKC- $\alpha$  phosphorylation after 40 minutes of exposure

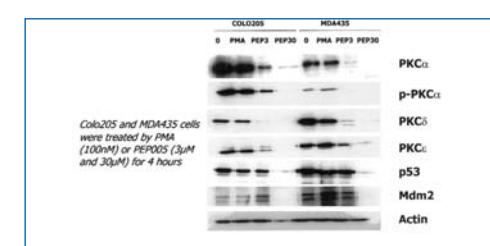
Translocation of PKC- $\delta$  in COLO-205 after 1 h PEP005 exposure



PEP005 induces activation of PKC- $\delta$  by translocation from the cytosol (C) to nucleus (N) and membranes (M)

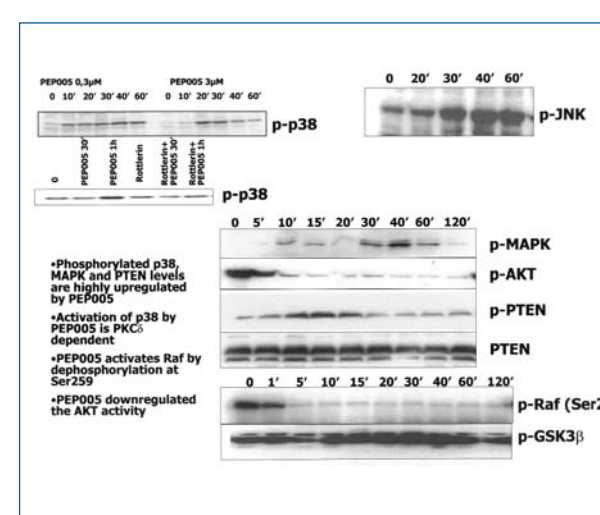
### 4 - PEP005 signaling pathways

Effects of PEP005 on PKC, p53 and Mdm2 expression



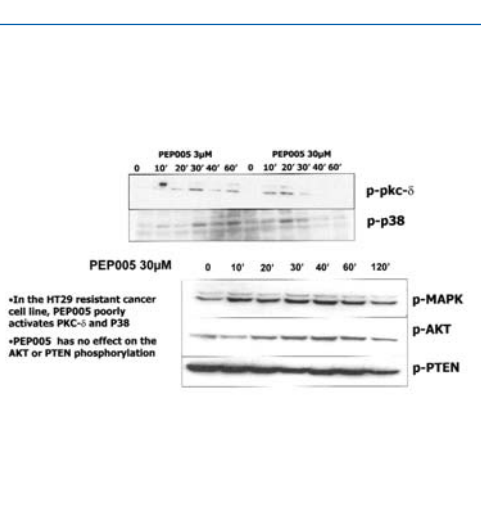
Protein level of PKC- $\alpha$ , p-PKC- $\alpha$ , PKC- $\delta$ , PKC- $\epsilon$ , p53 were down regulated by PEP005 in dose dependent manner

Effects of PEP005 on MAPK and AKT signaling pathways in COLO205 cells



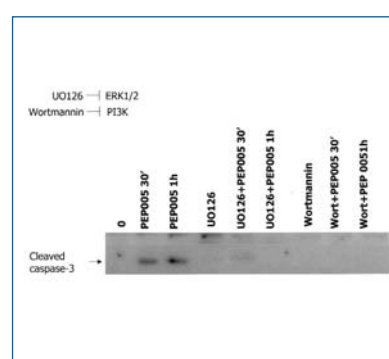
•Phosphorylated p38, MAPK and PTEN levels are highly upregulated by PEP005  
 •Activation of p38 by PEP005 is PKC-dependent  
 •PEP005 activates Raf by dephosphorylation at Ser259  
 •PEP005 downregulated the AKT activity

Effects of PEP005 on MAPK and AKT signaling pathways in HT29 cells

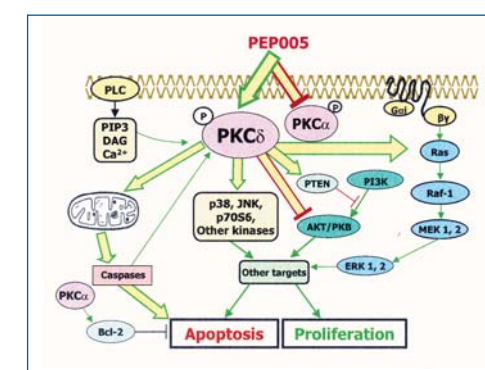


•In the HT29 resistant cancer cell line, PEP005 poorly activates PKC- $\delta$  and p38  
 •PEP005 has no effect on the AKT or PTEN phosphorylation

Effect of MAPK and PI3K inhibitors on PEP-induced caspase-3 activation



Proposed PEP005 signaling pathway



## CONCLUSION

- PEP005 may induce antiproliferatif effect by cell cycle arrest in S phase, apoptosis and necrosis. PEP005-induced apoptosis is caspase-3 dependent in COLO-205 cells.
- PEP005 is an effective activator of PKC- $\delta$  and PKC- $\delta$  translocation into nucleus and membranes. PEP005 also downregulates the expression and activity of PKC- $\alpha$ .
- PEP005 induced modulation of PKCs leads to Ras/Raf/MAPK and p38 activation and AKT/PKB inhibition.
- This study supports ongoing efforts targeting PKC isoforms in cancer therapy with PEP005 alone and in combination with other cytotoxic agents.

## References

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