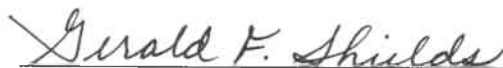


The Effect of PKC  $\delta$  Inhibitors on PMA-induced  
Apoptosis in *Xenopus laevis* Splenocytes

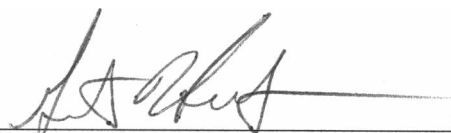
Submitted in Partial Fulfillment of the Requirements for Graduation  
With Honors to the Department of Natural Sciences at  
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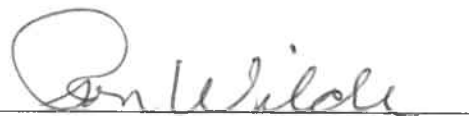
This thesis for honors recognition has been approved for the Department of  
Natural Sciences by:



Dr. Gerald Shields  
Thesis Director



Dr. Grant Hokit  
Reader



Dr. Ron Wilde  
Reader

April 7, 2003

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## Abstract

Protein kinase C (PKC) controls apoptosis in amphibian systems such as that of the South African clawed frog, *Xenopus laevis*. The effects of cancer inducing agents like phorbol 12-myristate 13-acetate (PMA) when combined with  $\text{Ca}^{2+}$  independent PKC inhibitors on programmed cell death were studied in *X. laevis* splenocytes. Regulation of PMA-activated cells was observed via Annexin V-FITC and propidium iodide assays, flow cytometry, and Western blot analysis. Results indicate the PKC inhibitors reduced both apoptosis and cell growth in PMA-stimulated *X. laevis* splenocytes compared to PMA alone. These results are valuable to future mammalian cancer research. By identifying the various apoptotic mechanisms utilized by amphibian cells, researchers may become more informed regarding amphibians' increased resistance to cancer and apply such knowledge to mammalian systems.

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## Introduction

### Importance of Apoptosis

Cell death, a normal physiological immune response, can be categorized into two distinct processes: necrosis and apoptosis. Necrosis, induced by chemical contamination, causes cellular inflammation and subsequent rupturing, infecting the surrounding medium with potentially harmful agents (Murphy *et al.*, 1990). In contrast, apoptosis, a nontoxic form of cell death, involves the phagocytic digestion of chemically tagged cells before they burst, disrupting neighboring cells (Pucci *et al.*, 2000). Distinguishing features associated with the apoptotic process include blebbing of the plasma membrane as well as DNA fragmentation (Duke *et al.*, 1996). As a whole, it is logical to assume that apoptosis is essential to the following cell functions: tissue homeostasis, removal of abnormal or cancerous cells, and protection from genetic errors inherent in development. Therefore, apoptosis plays a fundamental role in the regulation of the immune system. In fact, Duke *et al.* (1996) acknowledge that ineffective T-cells, those that lose the ability to locate and destroy foreign antigens, are eliminated via apoptotic means. With physiological processes contingent on apoptosis, particularly with regard to pathogenesis, it is imperative to understand the cellular aspects of programmed cell death.

Because apoptosis is vital to tissue homeostasis, it becomes prudent to understand apoptotic effects at the level of the cell cycle. While the cell cycle involves both DNA synthesis (S-phase) and the separation of two identical daughter cells (mitosis), the regulation “checkpoints” involved in the process are still unclear. It is known that growth factors are required for cells to progress to the next checkpoint; however, researchers note that the absence of growth factors does not always guarantee an arrest in the cycle

(Kroemer *et al.*, 1995). According to Kroemer *et al.* (1995), proteins involved in the cell cycle influence the sensitization of cells to apoptosis. By increasing or decreasing apoptotic susceptibility, proteins can directly affect the fate of a cell. Comparably, cell cycle manipulations have led to the presence or lack of apoptosis in some mammalian lymphocytes (Kroemer *et al.*, 1995).

Activation and inactivation of particular serine-threonine kinases, Cdks, regulate the cell cycle process (Lodish *et al.*, 1999). Because Cdks have been implicated in cell cycle control, it is reasonable to predict that they also serve an important role in apoptosis and cell proliferation. Indeed, studies involving mammalian thymocytes have indicated that apoptotic mechanisms and cell growth factors are both positively regulated by Cdk2 (Gil-Gomez *et al.*, 1998).

Due to the overall complexity of biological processes, it is logical to assume that the cell cycle is influenced by a multitude of contributory factors. For instance, Kong *et al.* (2000) discovered that the cell cycle is not only regulated by Cdks, it is also controlled by genetic components that additionally affect apoptosis. Researchers found that the Myc transcription factor, a product of the c-myc proto-oncogene, is involved in growth arrest of the cell cycle (Kong *et al.*, 2000). Others have observed the role of Myc in inducing cellular apoptosis as well (Pucci *et al.*, 2000). Pucci *et al.* (2000) postulate that the apoptotic condition is a default response in lieu of cellular proliferation. However, extensive research concerning apoptosis and the cell cycle is limited. Additional cell proliferation assays are needed to advance understanding of programmed cell death with regard to cellular growth.

Dysfunction of normal apoptotic mechanisms can lead to disease and ultimate death of the cell (Duke *et al.*, 1996). Presumably, variable apoptotic rates may influence both immune system response and tissue balance. For example, excessive apoptosis in helper T lymphocytes in the case of AIDS or the degeneration of neurons with respect to dementia are both examples of the impact of apoptosis on immunological functions (Duke *et al.*, 1996). In general, immune deficiencies associated with programmed cell death may be attributed to extreme apoptotic conditions. Contrary to the aforementioned case, other forms of affliction, including cancer, are the result of the cell's failure to induce apoptosis, allowing metastasis to occur (Duke *et al.*, 1996). Researchers acknowledge that current cancer treatments, like tamoxifen, impede cell cycle associated proteins and ultimately alter apoptotic functions (Duke *et al.*, 1996). However, the exact mechanisms cancer drugs utilize in apoptotic pathways are still unexplained. By focusing present immunological research efforts on apoptotic mechanisms, breakthroughs in cancer treatments may occur.

#### Role of Protein Kinase C (Effects of Activation/Inhibition)

To discern the exact relationship between apoptosis and malignancy, cellular components affecting apoptotic processes are studied. Phosphorylating enzymes, or kinases, have been found to play an integral role in transmembrane signaling (Lodish *et al.*, 1999). Generally, the process of phosphorylation changes a protein's charge and thus, ultimately influences the enzymatic activity of the intended protein. Specifically, apoptotic regulation is mediated by a specific serine-threonine kinase known as protein kinase C, or PKC (Dekker and Parker, 1994). PKC is composed of eleven closely related isoforms, classified according to their calcium dependency. The Ca<sup>2+</sup> dependent

isoforms, including  $\alpha$  and  $\beta$ , rely on the calcium cation for enzymatic activity (Dekker and Parker, 1994). Unlike the  $\text{Ca}^{2+}$  dependent isoforms, PKC  $\delta$  and  $\epsilon$  do not require calcium to sustain cellular activities (Dekker and Parker, 1994). The differential  $\text{Ca}^{2+}$  dependency may suggest that the distinct isoforms maintain unique functions within the cell; however, inadequate supportive evidence requires further research to substantiate such claims.

In addition, the aforementioned PKC isoforms are selectively activated by certain phorbol esters. One such ester, phorbol 12-myristate 13-acetate (PMA), selectively activates the following PKC isoforms:  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\epsilon$ . PMA has been shown to cause a cytoprotective effect in most organisms (Gomez-Angelats *et al.*, 2000). For instance, mammalian studies indicate that the inhibition of PKC  $\alpha$  and  $\beta$  plays a fundamental role in the failure of PMA to induce apoptosis (Gomez-Angelats *et al.*, 1999).

While PKC  $\alpha$  and  $\beta$  are associated with anti-apoptotic activity, PKC  $\delta$  maintains a pro-apoptotic function in both mammalian and amphibian cells (Fujii *et al.*, 2000). Fujii *et al.* (2000) have indicated that PKC  $\delta$  increases apoptosis in malignant human prostate cells. It is assumed that the resultant apoptotic rise is correlated with a reduction in neoplastic transformation or cancer incidence.

With implications of PKC  $\delta$ 's importance in apoptosis, it is imperative to explore the ways in which inhibitory compounds affect such a process. One such inhibitor, GF109203X (GF), suppresses PKC  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\epsilon$  (McMahan *et al.*, 1999). Counteracting the effects of PMA, GF provides valuable insight to quantifying the true connection between apoptosis and cell proliferation.

Likewise, cycloheximide has been known to prevent the synthesis of pro-apoptotic proteins and, consequently, suppress apoptosis (Murphy *et al.*, 1990). However, others implicate cycloheximide in the synthesis of anti-apoptotic proteins, decreasing apoptotic rates (Ruben *et al.*, 2000).

Another PKC  $\delta$  inhibitor, rottlerin, also inhibits apoptotic activity (Lu *et al.*, 1997). According to Lu *et al.* (1997), when rat fibroblasts are treated with rottlerin, cellular metastasis occurs. Therefore, the researchers implicate PKC  $\delta$  serving an integral role in pro-apoptotic functioning.

Interestingly, Ruben *et al.* (2000) contend that the individual inhibitors, GF, cycloheximide, and rottlerin, induce apoptosis on their own, but when combined with an activator they exhibit inhibition. Coupling an activator, such as PMA, to various PKC  $\delta$  suppressors allows researchers to decipher PMA's collective effect on programmed cell death as well as cellular growth.

#### *Xenopus laevis* and Apoptosis

Previous scientific data show that amphibian cells have an increased susceptibility to apoptosis and a decreased vulnerability to cancer. When injected with cancer-inducing endogens, the frequency of neoplastic transformation in the South African clawed frog, *Xenopus laevis*, is significantly low (Clothier *et al.*, 1989). Additionally, Ruben *et al.* (2000) proposed that *Xenopus* splenocytes are, in fact, "exquisitely sensitive to reagents that affect PKC activity."

Though mammals and amphibians may hold differential susceptibilities to PKC  $\delta$  inhibitors, such as cycloheximide and rottlerin, it is likely that the general apoptotic components of each organism are evolutionarily conserved (McMahan *et al.*, 1999).

Thus, it is advantageous to compare both the mammalian and amphibian immune systems with regard to apoptosis. In particular, differences in PMA-induced apoptosis suggest variable pathways for programmed cell death in mammals and *X. laevis* (Ruben *et al.*, 2000).

While the cancer connection to apoptosis is highly researched via individual PKC  $\delta$  activators and inhibitors, scientists have yet to evaluate cell growth within the context of programmed cell death. It is presumed that cell proliferation decreases as apoptosis increases. But, the exact relationship between cell growth and apoptosis has yet to be characterized.

Therefore, a potential key to understanding the amphibian resistance to cancer may lie in determining the influence of PMA on both apoptotic mechanisms and cellular growth. While the next logical step in solving the apoptotic puzzle is to determine the exact relationship between PKC  $\delta$  stimulators, suppressors, and their collective effect on programmed cell death, before such experiments are implemented, it is essential to review the precise role of PMA in apoptosis and cell proliferation. Based on the aforementioned research, I specifically hypothesized that the phorbol ester, PMA, will exclusively affect either apoptosis or cell growth in *X. laevis* splenocytes. Particularly, I predicted that cell proliferation decreases as apoptosis increases.

## Materials & Methods

### Laboratory Animals

The South African clawed frog was utilized for all experimental assays. All *X. laevis* were reared in 100 gallon stock containers (25 *X. laevis*/container) filled halfway with oxygenated de-ionized water at 23 °C. In addition, each container was fitted with a rudimentary mesh screen to prevent escape. The toads were fed frog brittle (NASCO, Oshkosh, WI) and kept in accordance with the NIH guidelines for the Care of Vertebrate Experimental Animals.

### Splenocyte Cultures

Randomly chosen adult *X. laevis* were submerged in KMnO<sub>4</sub> solution (15 minutes) for sterilization purposes. Anesthetization of the animals in MS-222 for five to ten minutes ensued. Prior to anesthetization, MS-222, composed of 1.34 g ethyl-m-aminobenzoate in 100 mL nanopure water, was stored at -20 °C (Fisher Scientific, Pittsburgh, PA). Sterile technique was then followed to extract *X. laevis* spleens. The extracted spleens were placed in 3 mL of culture medium comprised of one package Leibovitz L-15 medium (Sigma, St. Louis, MO), 0.021 g streptomycin, 0.055 g penicillin, 2.97 g HEPES powder in 1.5 L nanopure water, and 10% fetal bovine serum at pH 7.4 (Thronson, 2001). The frog splenocytes were subsequently dissected with sterile forceps, suspended in 15 mL centrifuge tubes, and centrifuged at 700 x g for 5 minutes. Upon removal of the supernatant, which consisted of cellular debris, the splenocytes were resuspended in 1 mL of the aforementioned culture medium. A hemocytometer was then utilized for cell counts of 15 µL aliquots. Once counted, the splenocyte suspensions were adjusted to a concentration of 10<sup>6</sup> cells per mL. One hundred µL of cell suspension

combined with 100  $\mu$ L of control medium or treatment were pipetted into individual wells of a 96-well round-bottom plate and cultured for 72 hours.

#### Treatment Cultures

PMA treatment wells were maintained at a concentration of 50 ng/mL [1 mg/mL PMA in DMSO stock solution, diluted with L-15 culture medium (Thronson, 2001)]. In-well concentrations of rottlerin treatments were 0.1  $\mu$ g/mL (+/- PMA), 0.5  $\mu$ g/mL (+/- PMA), 1  $\mu$ g/mL (+/- PMA), 2  $\mu$ g/mL (+/- PMA), and 4  $\mu$ g/mL (+/- PMA). Finally, cycloheximide treatments were prepared at concentrations of 7 ng/mL (+/- PMA) and 10 ng/mL (+/- PMA).

#### Western Blot Analysis

According to Pierce's (Rockford, IL) protocol, the NE-PER extraction kit was used to prepare nuclear and cytoplasmic extracts, which were stored at -20 °C in 15  $\mu$ L aliquots.

Each lane of a 10% SDS-polyacrylamide mini gel (BioRad, Hercules, CA) was loaded with 15  $\mu$ L of extract. One lane contained a BioRad kaleidoscope molecular marker. Electrophoresis of the samples ensued for 1 hour at 100 V.

Proteins in the gel were then transferred to nitrocellulose or PVDF membrane (BioRad, Hercules, CA) for 1 hour at 52-72 V. The PVDF membrane was stained in Ponceau S. A solution of 1X TBS with 5% non-fat dried milk (BioRad, Hercules, CA) was used to rinse and block the membrane for 24 hours. The membrane was rocked and kept at 4 °C for the duration of the blocking. After rinsing 3 times in TTBS (1X TBS, 0.05% TWEEN 20), the membrane was incubated at room temperature for 1 hour in a concentration of primary antibody ranging from 1.6-6.6  $\mu$ g/mL. Again, the membrane

was rinsed 3 times with TTBS and incubated at room temperature for 1 hour in horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Santa Cruz, CA). TTBS was then used to wash the membrane once for 15 minutes, once for 10 minutes, and twice for 5 minutes. Finally, the membrane was saturated with 4 mL chemilluminescence reagent (NEN Life Science Products, Boston, MA) for 1 min, allowed to dry, and exposed to Kodak Bio-Max X-Ray film in the dark.

#### Annexin V-FITC and Propidium Iodide Assays

Cell treatments were transferred from the round-bottom well plate, following culture, to Eppendorf tubes containing 10  $\mu$ L Media Binding Reagent and 1.25  $\mu$ L Annexin V-FITC. Individual samples were placed in the dark for 15 minutes at room temperature to incubate. After incubation, the tubes were centrifuged at 1000 x g for 5 minutes and the pellet was resuspended in 0.5 mL cold 1X Binding Buffer along with 10  $\mu$ L propidium iodide, PI. All samples were iced until further analysis by flow cytometry. All reagents used for the apoptosis and proliferation assays comprised the Annexin V-FITC Apoptosis Detection Kit (Oncogene Research Products, Cambridge, MA).

To quantify the staining of each cell treatment, fluorescence of Annexin V-FITC at 518 nm and PI signals of 620 nm were used. Ten thousand total gated events were counted for each treatment and control group.

#### Flow Cytometry

A flow cytometer utilizing an argon ion laser light with a wavelength of 488 nm was used to quantify the Annexin V-FITC and PI assays. Oregon Health Sciences University (Portland, OR) performed all flow cytometry assays.

### Statistical Analysis of Proliferation Assays

Significance of treatment effects was determined by analyses of variances (ANOVAs). Confidence intervals of 95% correspond to error bars. A 5% confidence interval ( $p < 0.05$ ) indicates statistical significance between treatments. StatView 5.0 (Abacus Concepts, Palo Alto, CA) was used for all statistical analyses.

## Results

### Western Blotting & Chemilluminescence

The units of the standard for the Western blot are 42 kDa. The Western blot performed on *X. laevis* splenocytes shows a clear protein band present at 80 Da (Figure 1). When PKC is activated by PMA alone, the protein band is absent. However, when combined with GF, an inhibitor of PKC  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\epsilon$ , PMA exhibits the 80 Da band.

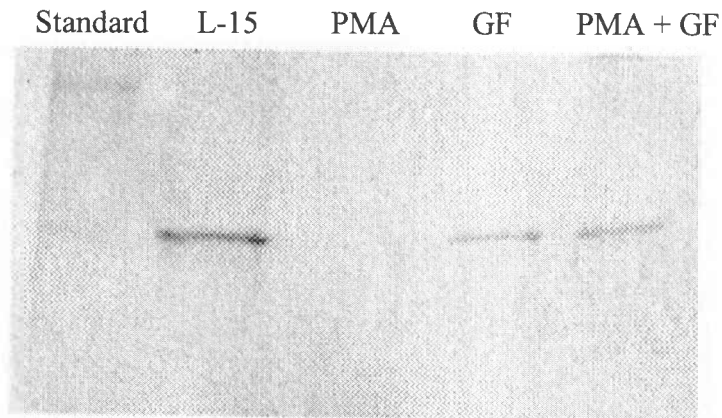


Figure 1: Anti-PKC  $\delta$  80 Da Western Blot Protein Band

### Annexin V-FITC & Propidium Iodide Staining

The following Annexin V-FITC bit maps indicate a statistically significant decrease in the number of cells gated in late apoptosis for PMA activated treatments (Figure 2). When compared to the control in Figure 2, PMA alone decreased late apoptosis by 1.73%. In addition, early apoptosis increased 16.55%. Additional treatments such as PMA + cycloheximide (CHX) and PMA + rottlerin (ROTT) also illustrate the trend associated with PMA exposure.

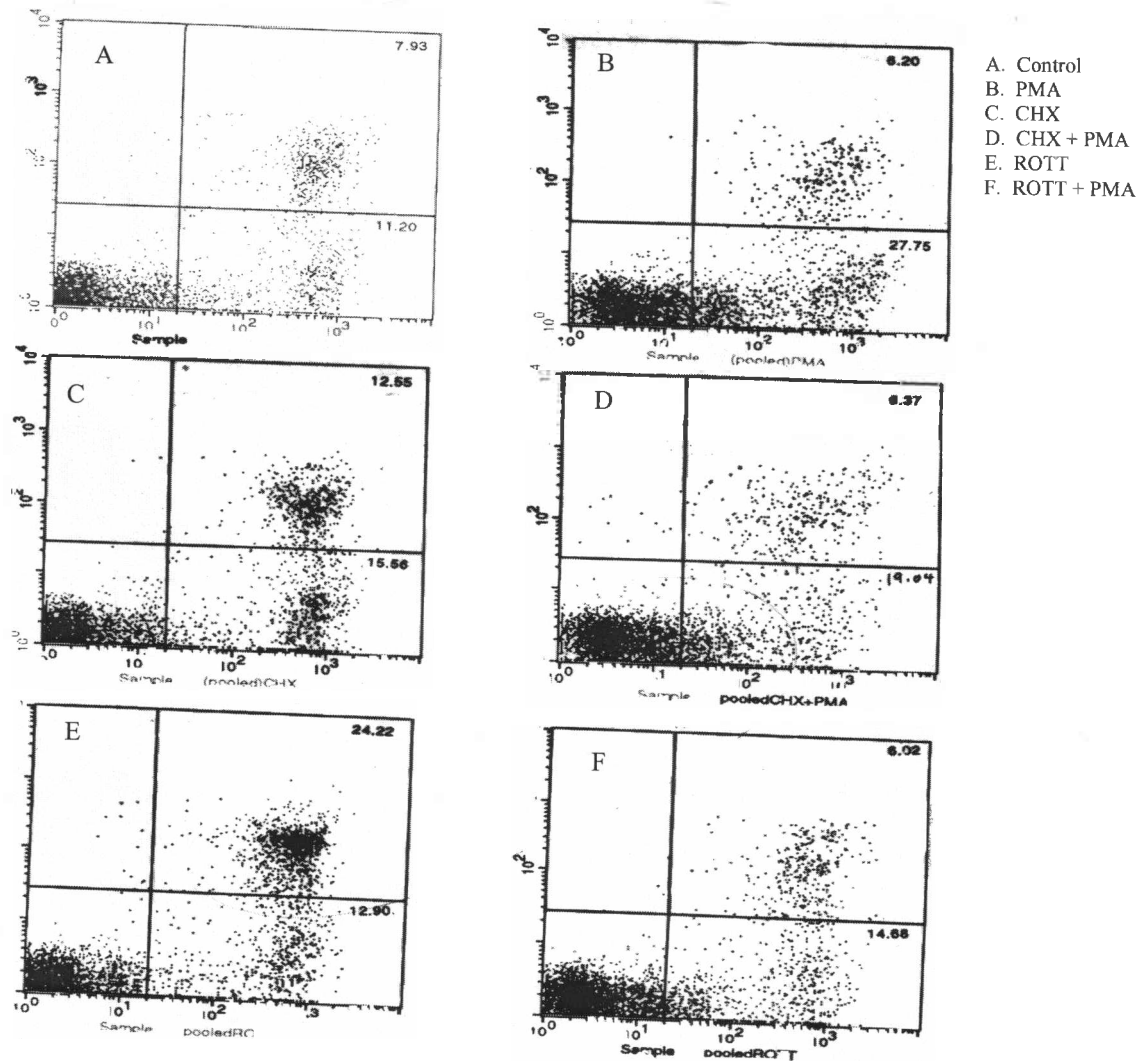


Figure 2: Annexin V-FITC Bit Maps

Analyses of Variances (ANOVAs) were performed on the Annexin V-FITC and PI results. As illustrated in Figure 3, when compared to the control, PMA shows statistical significance with a p-value < 0.0001. Similarly, compared to PMA alone, both CHX and PMA + CHX treatments maintain statistical significance (p-value < 0.0001).

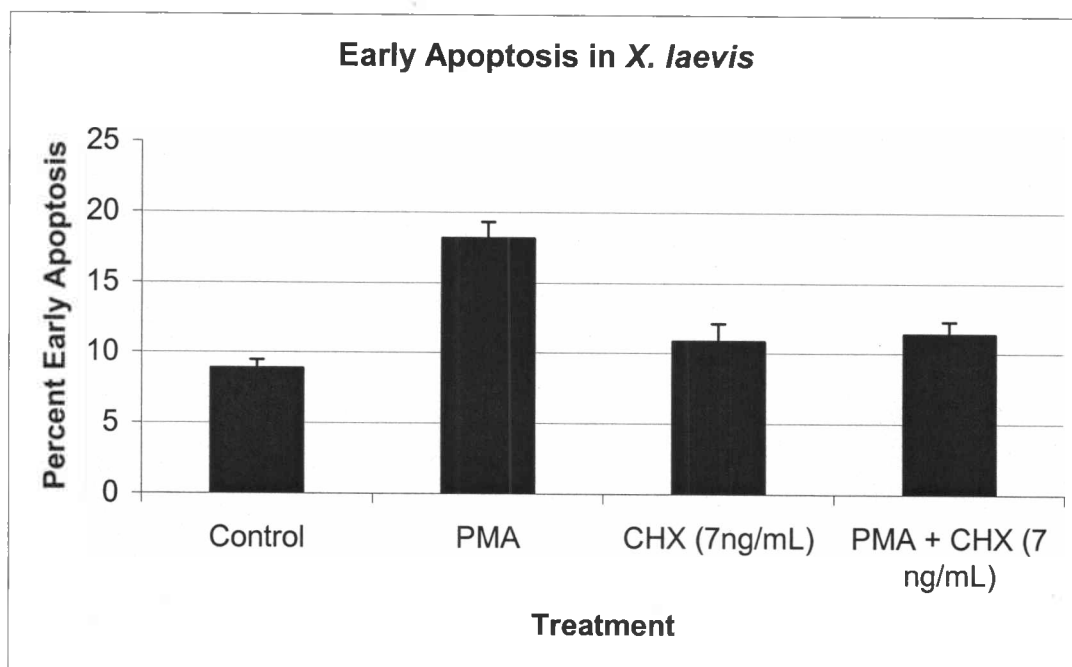


Figure 3: Effect of Cycloheximide & PMA on Early Apoptosis in *X. laevis* Splenocytes  
Flow Cytometry

Figure 4 displays the resultant flow cytometry bit maps. The number of enlarged cells is denoted by the R3 gated region for each treatment condition. The effect of cycloheximide and PMA on enlarged *X. laevis* splenocytes is shown in Figure 5.

Statistically significant treatments include the following: PMA when compared to the control group (p-value = 0.0013), PMA in contrast to CHX (p-value = 0.0002), and PMA alone compared to the PMA + CHX combination (p-value = 0.0348).

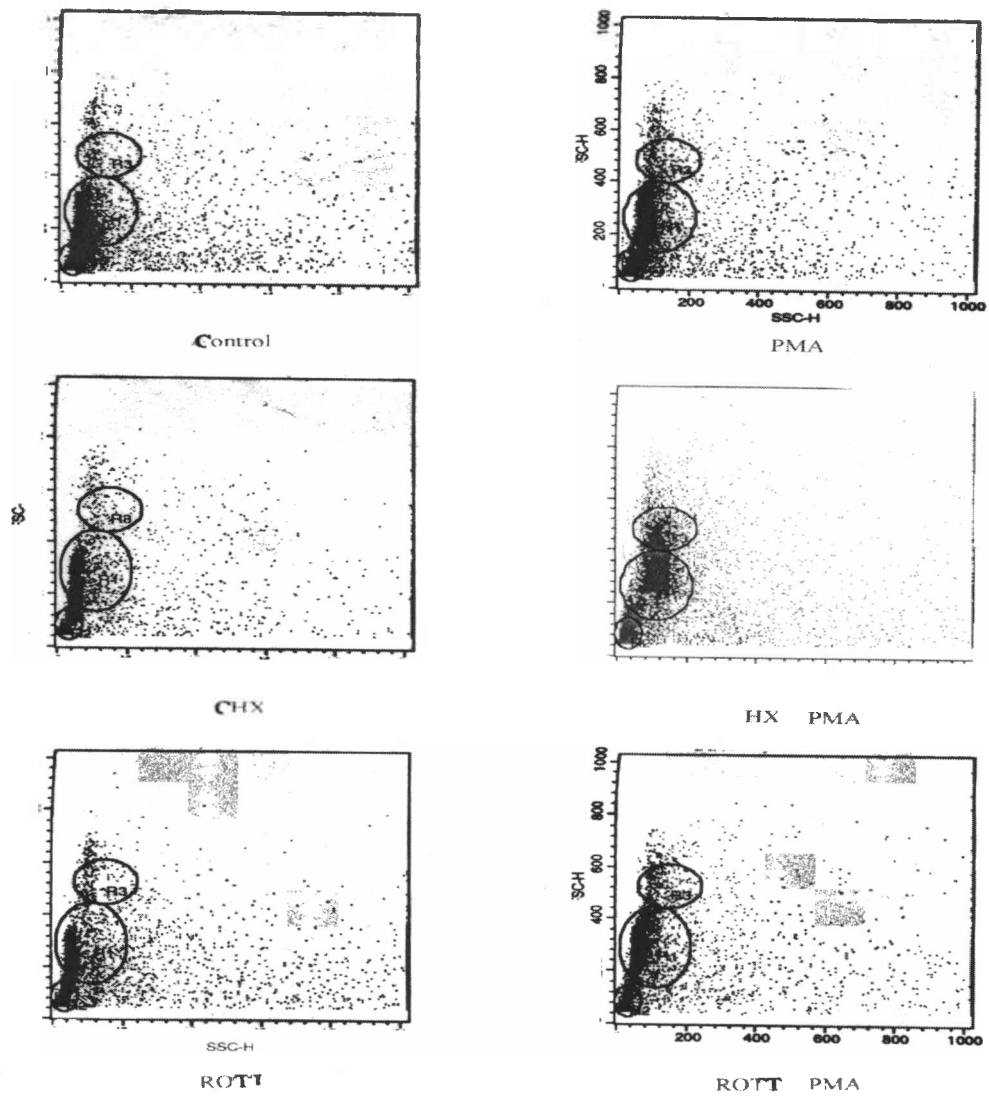


Figure 4: Flow Cytometry Bit Maps

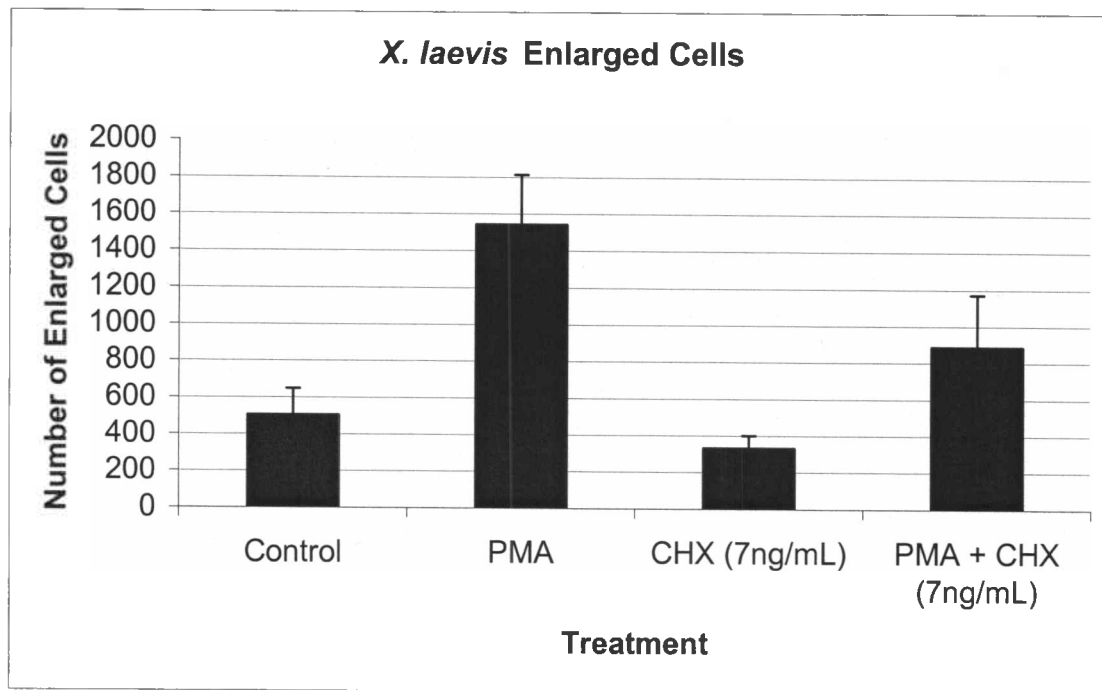


Figure 5: Effect of Cycloheximide & PMA on Enlarged Cells of *X. laevis*

The PKC  $\delta$  inhibitor, rottlerin, produced identical Annexin V-FITC, PI, and flow cytometry results. Statistical significance was computed at p-values  $< 0.0001$  for the same treatment conditions as CHX.

## Discussion

### GF109203X

Western blot analysis substantially reinforces the aforementioned apoptotic trend (Figure 1). Interestingly, GF alone produces an 80 Da protein band. Yet, when combined with PMA, the protein band is no longer visible. Based on the Western blot results, GF, a general PKC inhibitor, may be utilizing a separate apoptotic pathway from PMA. However, even though these findings indicate that inhibitors, such as GF, fail to involve PKC in their respective apoptotic pathways, data obtained from Annexin V-FITC bit maps suggest otherwise (Figure 2). Indeed, the results indicate a reliance of the various inhibitors on PKC-dependent apoptotic pathways.

### Cycloheximide

The statistical significance ( $p < 0.0001$ ) of cycloheximide treatments when compared to controls suggests the reagent's inherent role in PMA-induced apoptosis. An inhibitor of PKC  $\delta$  and  $\epsilon$ , cycloheximide has previously been shown to increase apoptotic rates in mammalian B-lymphocytes (Lu *et al.*, 1997). Similarly, *X. laevis* splenocytes treated with the reagent generate comparable results.

Alternatively, studies conducted by Murphy *et al.* (1990) support the reagent's ability to suppress apoptosis. The drastic difference in reported results may be attributed to the dose-dependent nature of cycloheximide. Thronson (2001) demonstrates that cycloheximide concentrations greater than 10 ng/mL induce apoptosis while concentrations significantly below this value inconclusively suggest neither pro-apoptotic nor anti-apoptotic trends.

Moreover, as illustrated in Figure 2, cycloheximide stimulates considerably more late apoptosis than PMA alone. Although cycloheximide inhibits PMA induced early apoptosis, at the same time, PMA appears to “protect” cells from cycloheximide induced late apoptosis. Overall, the resultant combination of PMA + CHX reduces apoptosis in *X. laevis* splenocytes.

Comparably, Figure 4 shows a significant increase in the number of enlarged cells for PMA treatments when compared to the control. However, the cellular growth inherent in the PKC  $\delta$  activator’s treatment seems to be alternatively affected by the addition of cycloheximide. The reversal in cell proliferation is a clear result of the combined PKC  $\delta$  activator and suppressor treatment. Because the response in early apoptosis and the number of enlarged cells to PMA and PMA plus a PKC  $\delta$  inhibitor is similar, apoptosis and cell growth appear to be positively correlated. Therefore, I reject my original hypothesis due to the fact that when PMA is suppressed with either cycloheximide or rottlerin, both cell growth and apoptosis are blocked.

#### Rottlerin

Studies of cycloheximide and rottlerin have shown that rottlerin’s effect on PKC  $\delta$  is directly implicated in pro-apoptotic pathways (Lu *et al.*, 1997). Others have also supported the role rottlerin plays in increasing cell death by researching high PKC activity of *X. laevis* splenocytes (Ruben *et al.*, 2000).

As Figure 2 suggests, rottlerin does indeed play a substantial role in PMA-induced apoptosis. Similar to cycloheximide, rottlerin displays considerably more late apoptosis compared to PMA alone. Because PMA stimulates significantly more early apoptosis while rottlerin primarily stimulates late apoptosis, this differential apoptotic

selection may be the key to understanding the uncertain phenomenon when the two are combined. Again, the combination of PMA and a PKC  $\delta$  inhibitor seems to produce identical results: PMA reduces rottlerin's ability to stimulate late apoptosis while rottlerin inhibits PMA's early apoptotic effects.

With regard to cell growth, rottlerin combined with PMA induces a similar decrease in the number of enlarged cells as the CHX + PMA treatment (Figure 4). Thus, in *X. laevis* splenocytes, PMA activates both cell growth and apoptosis. An explanation for the aforementioned contradictory findings may be that individual cells respond to PMA differently depending on their location in the cell cycle. Additionally, a cell's general physiological state might contribute to the observed response.

#### Future Cancer Treatments

Mammalian cancer research may benefit from analogous *X. laevis* PKC data by focusing on the differential apoptotic mechanisms utilized by amphibian cells. This regulation of both programmed cell death and proliferation through PKC is the fundamental barrier blocking current understanding of neoplastic transformations. When cancer-inducing agents, such as PMA, are introduced to *X. laevis* splenocytes, both apoptosis and cell growth increase. Therefore, creating an experiment to identify the various cellular mechanisms contributing to the observed amphibian cancer-resistance would prove beneficial to mammalian cancer studies.

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